
Biom mineralization – Medical Aspects of Solubility

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Erich Königsberger and LanChi Königsberger

Murdoch University, Perth, WA, Australia



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Library of Congress Cataloging in Publication Data

Biomineralization : medical aspects of solubility / [edited by] Erich Königsberger and
LanChi Königsberger.

p. ; cm.

Includes bibliographical references and index.

ISBN-13: 978-0-470-09209-5 (alk. paper)

ISBN-10: 0-470-09209-2 (alk. paper)

1. Biomineralization. I. Königsberger, Erich. II. Königsberger, LanChi. III. Title.

[DNLM: 1. Calcification, Physiologic. 2. Minerals. 3. Solubility. WE 200 B6157 2006]

QH512. B565 2006

612'. 01524—dc22

2006005377

British Library Cataloguing in Publication Data

A catalogue record for this book is available from the British Library

ISBN-10 0-470-09209-2

ISBN-13 978-0-470-09209-5

Typeset in 10/12pt Times by Integra Software Services Pvt. Ltd, Pondicherry, India

Printed and bound in Great Britain by TJ International, Padstow, Cornwall

This book is printed on acid-free paper responsibly manufactured from sustainable forestry in which at least two trees are planted for each one used for paper production.

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Foreword

Biom mineralization – Medical Aspects of Solubility is an entirely unpretentious, down-to-earth title for a scientific treatise of respective phenomena. Beneath this plain surface, however, there hides an ambitious and meritorious attempt to bridge the gap between two highly developed and definitely distinct academic cultures, namely the medical and the physicochemical ones. That these cultures necessarily tend to develop in different directions can easily be illustrated by examples.

An exemplary situation in medicine is that penicillin on the one hand saved millions of lives, but in rare cases, on the other hand, a penicillin allergy can cause a lethal anaphylactic reaction. In physicochemical equations every quantity is individually measurable, and in an adequately equipped laboratory every scientist must, for example, be able to measure the same numerical value for a well-defined solubility. In medicine repeatability of a therapeutic success can only be hoped for with a certain probability, whereas in physical chemistry irreproducible measurements mean unequivocally that something is wrong.

How then can the gap be bridged between medical concepts of biom mineralization and physicochemical concepts of solubility? Medicine can learn from physical chemistry to use, as much as possible, straightforward mathematics to predict therapeutic consequences. Physical chemistry can learn from medicine which complicated systems are worthwhile to be investigated and which data important for medical predictions should urgently be made available.

Everybody successful in such an enterprise has to be sufficiently familiar with two different academic cultures, but then this qualification guarantees an impressive impact on science. For me it is a personal satisfaction that the Königsbergers' activities within the IUPAC Solubility Data Group stimulated the edition of this book, which provides excellent reading. I congratulate the authors of this volume on their courage to perform and describe research at the boundary between medical and physicochemical sciences.

Heinz Gamsjäger

Preface

This book was initiated through discussions with Dr Peter Fogg, the then editor of the Wiley Series in Solution Chemistry, who asked us to edit a book on medical aspects of solubility for the (meanwhile discontinued) series. In various meetings of the IUPAC Solubility Data Commission V.8 (now IUPAC Subcommittee on Solubility and Equilibrium Data) this idea was developed further and supported particularly by Prof. Heinz Gamsjäger, the current Chairman of SSED. We are grateful to Dr Fogg and Prof. Gamsjäger for their encouragement and advice during the early phases of this project.

Solubility phenomena are fundamental to living organisms and range from gas solubilities (e.g. oxygen in blood) to biomineral formation in body fluids. This book comprises five chapters on various perspectives of normal and pathological biomineralization in humans. Chapter 1 gives an overview of experimental and modeling methods, recommends solubility data for selected stone-forming substances and describes recently discovered, unusual dissolution and crystallization phenomena involving nano-sized biomaterials. Chapter 2 presents a general classification of renal and salivary calculi based on their formation mechanism. Chapter 3 reviews the solubilities of calcium and magnesium phosphates and discusses their relevance to normal and pathological mineralization in terms of stability field diagrams. Chapter 4 proposes a new paradigm for biomineralization – liquid phase precursors – and their possible roles in the formation of bone and kidney stones. In Chapter 5, various aspects of the biomineralization of iron and its relation to iron-overload diseases are discussed. Rather than giving purely phenomenological descriptions, the results and conclusions presented in this book are frequently based on quantitative physicochemical measurements.

Erich and LanChi Königsberger

Acknowledgements

We thank all contributors for their effort and care in preparing their chapters. Special gratitude is due to Miss Jenny Cossham, Mrs Lynette James and Miss Zoe Jenner of Wiley, Miss Vidya Vijayan of Integra Software Services who have been responsible for the publication of this book in various stages of its production. Their readiness to help with all major and minor issues and their patience with tardy editors is greatly appreciated.

Erich and LanChi Königsberger

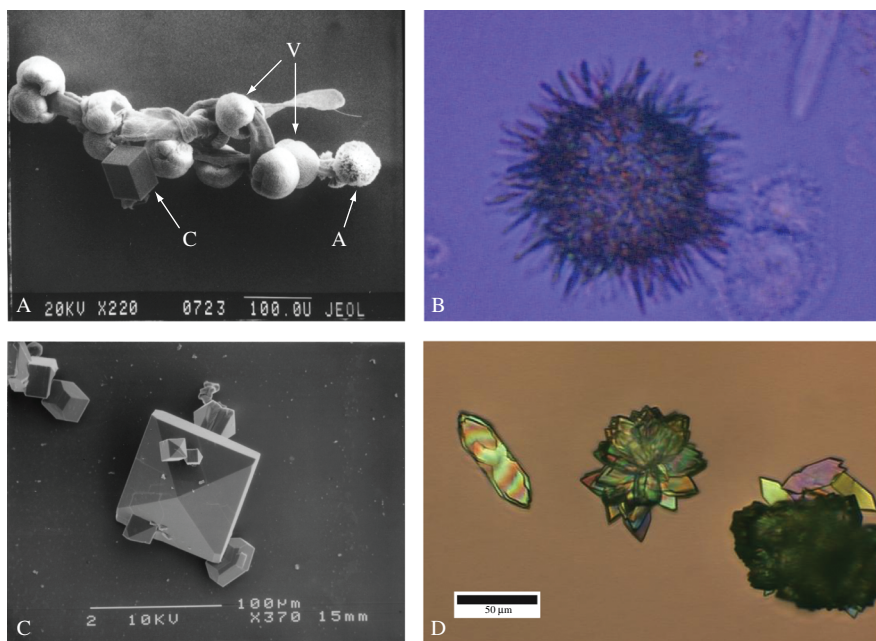


Plate 1 (Figure 4.3) The inorganic crystal habits of the three calcium-based minerals that are commonly found in biological hard tissues. (A) This SEM micrograph shows all three anhydrous phases of calcium carbonate which nucleated on a piece of dust in the crystallizing solution. Calcite is seen as the well faceted rhombohedron (indicated by C), an aragonite spherulite composed of orthorhombic needles is marked with an A and the donut-shaped spherulites are vaterite, marked with a V. (B) Calcium phosphate commonly forms spherulitic clusters of hydroxyapatite needles. This spherulite was originally an amorphous globule (like the one to the bottom right), which then recrystallized into needles of HA. (C) Calcium oxalate most commonly occurs as tetragonal bipyramids of calcium oxalate dihydrate (COD), or (D) as coffin-shaped monohydrate (COM) crystals which are often twinned. Excessive twinning leads to clusters and spherulites, as seen here.

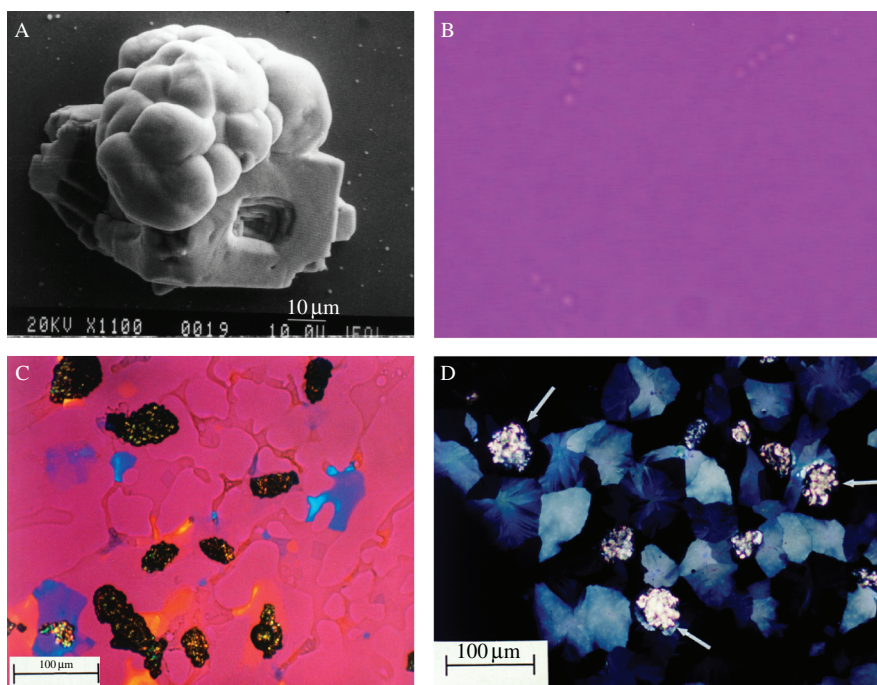


Plate 2 (Figure 4.5) Calcium carbonate morphologies produced during the PILP process. (A) With low levels of polyaspartate additive, aggregates of rounded crystals are produced. The round crystals have been determined to be spherulites of vaterite, which are smooth because they are coated with a 'membrane' of mineral film. Even the faceted rhombohedral crystals of calcite appear distorted, and are considered hybrid crystals, which are presumably formed from the traditional solution growth, but become distorted by the presence of the fluidic amorphous precursor. (B) Droplets of the PILP phase can be seen in the early stage by *in situ* examination of the reaction when the solution becomes cloudy. Aggregation of the droplets is often observed, and here the droplets ($\approx 2\mu\text{m}$ in diameter) appear to be congregating into linear chains. (C) The precursor phase has deposited as a swirly film on the glass coverslip, and is beginning to transform. The amorphous films appear the same magenta color as isotropic background, while the orange and blue (go gators!) patches are single-crystalline calcite. Many aggregates are also present, and appear dark since they are much thicker than the thin films. (D) After transformation, the films appear fully birefringent, except the patches that are oriented in the extinct position. The arrows point to aggregates of calcite, which are nearly always present unless the reaction is performed at low temperature (4°C), or in the presence of Mg ion additive. (Reprinted from *Journal of Crystal Growth*, Vol 191, Gower LA, Tirrell DA, Calcium Carbonate Films and Helices Grown in Solutions of Poly(aspartate), 153–160, Copyright 1998, with permission from Elsevier.)

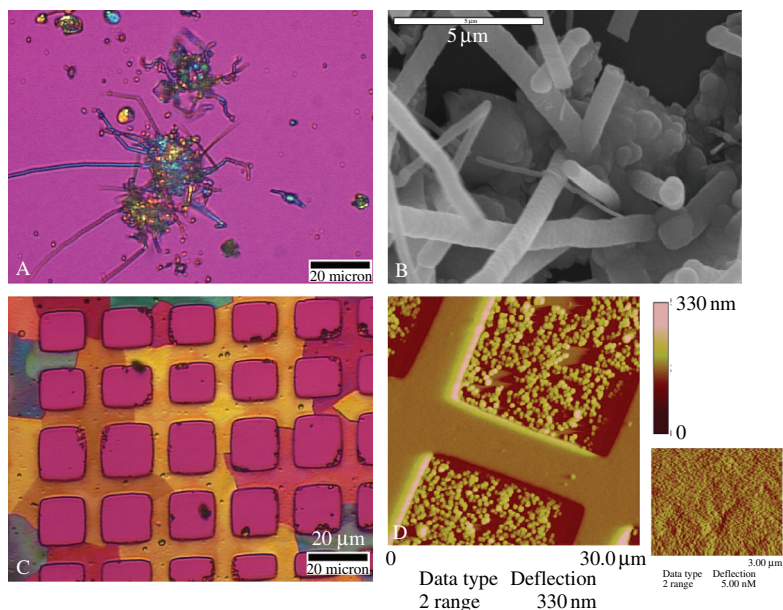


Plate 3 (Figure 4.7) Examples of nonequilibrium crystal morphologies produced for calcite crystals formed by the PILP process. (A) Fibers of calcite were ‘extruded’ from an amorphous globule of PILP phase. The uniform retardation color indicates they are single-crystalline (as well as electron diffraction spot patterns of isolated fibers). (B) Scanning electron microscopy shows the structure of the fibers, which clearly are not needles (as might be found for aragonite). Note, the small fiber in the middle is ‘draped’ across the larger fiber, suggesting that the shape was formed before the precursor material fully solidified. (For Figures 4.7A and B, Copyright 2003 from A New Paradigm for Biomineral Formation: Mineralization via an Amorphous Liquid-Phase Precursor, *Connective Tissue Research* 44 (Suppl. 1): 326–334 (2003) by Olszta MJ, Odom DJ, Douglas EP, Gower LB. Reproduced by permission of Taylor & Francis Group, LLC., <http://www.taylorandfrancis.com>.) (C) The soft lithography technique of microcontact printing was used to pattern a gold-coated substrate with self-assembled monolayers (SAMs) of alkane thiols. The PILP phase preferentially deposited on the carboxylate terminated SAMs, yielding a patterned film of calcite. In essence, single-crystalline patches of calcite were ‘molded’ by spatial delineation of the templating substrate. (Reproduced by permission of Kim Y, Gower LB, Formation of Complex Non-Equilibrium Morphologies of Calcite via Biomimetic Processing, *Materials Research Society Symposium Proceedings* 774: 141–148 (2003).) (D) Atomic force microscopy deflection images demonstrate that the patterned calcite films were generated from colloidal droplets of the precursor phase. Large droplets sometimes deposit in the nonpreferred region, as can be seen in the middle of the grid pattern. These droplets were more solidified than the fine-scale colloids that deposited earlier in the preferred region (inset at the bottom right was taken from the smooth grid region of calcite).

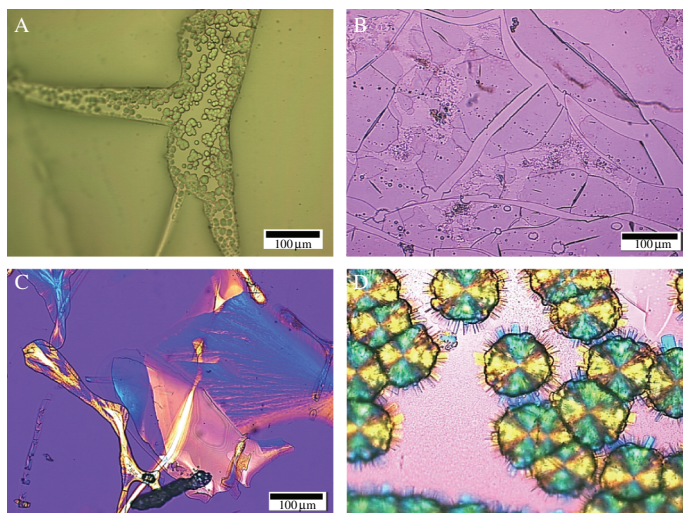


Plate 4 (Figure 4.8) Structures and transformation of amorphous calcium carbonate (ACC). (A) Coalesced droplets of PILP phase can be seen at the edge of this spongy looking film, which apparently contains much more water than the glassy ACC film shown in (B). Both of these films were formed at the air–water interface under Langmuir monolayers of steric acid. The film in (B) cracked as it was dipped off the air–water interface onto a glass slide, showing the brittle nature of its glassy composition. (C) This ACC film is beginning to transform by the pseudo-solid-state crystallization mechanism. The solid orange-yellow region is transforming into single-crystalline calcite, while the striped region is transforming into spherulitic vaterite (as a two-dimensional spherulitic film). (D) This film seems to be transforming via dissolution and recrystallization into three-dimensional spherulites, although the outer edges of the spherulites appear to be crystallizing by the pseudo-solid-state transformation.

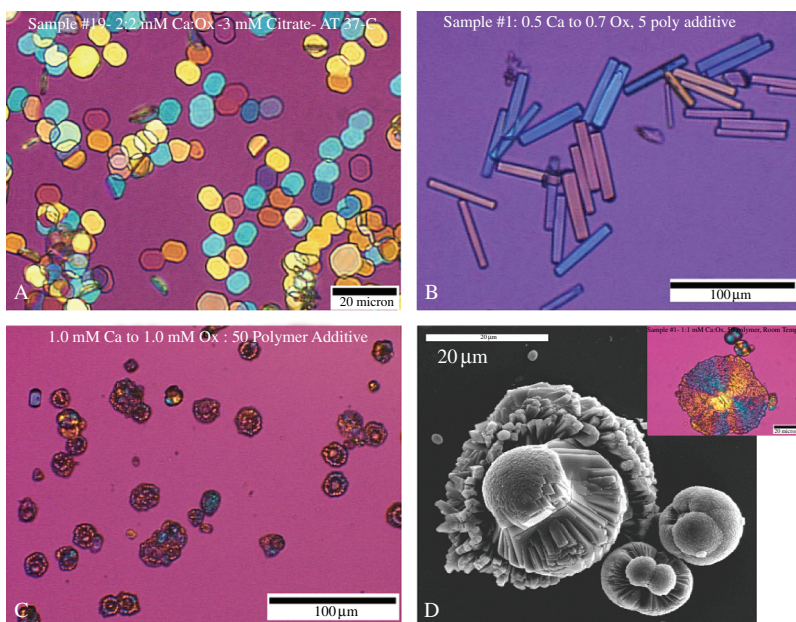


Plate 5 (Figure 4.9) Variations in calcium oxalate morphologies produced with simple crystal growth modifiers. The crystal phase has not been determined by X-ray diffraction, but they appear to all be modified COM crystals, which normally exhibits the coffin-shaped morphology (Figure 4.3D) under similar conditions, but without additive. (A) With 3 mM citrate, thin flat platelets with very rounded ends are produced. (B) Elongated prisms without triangular or rounded ends are produced with a small amount (5 µg/ml) of polyaspartic acid. (C) A higher concentration of the polymer (50 µg/ml) leads to unusual knobby aggregates, which likely recrystallized from an amorphous globule. (D) A polarized micrograph at higher magnification (inset at top right) shows the distinct Maltese cross pattern of these spherulitic aggregates, and the scanning electron micrograph shows the formation of asymmetric spherulites, which is the cause of the knob at the middle of each globule.

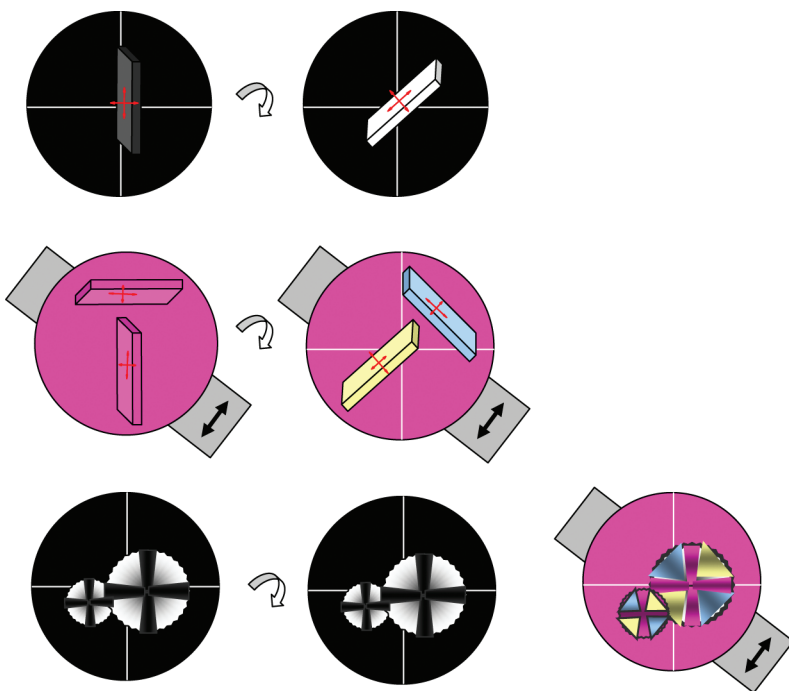


Plate 6 (Figure 4.38) Birefringence patterns of crystals with polarized light microscopy. (A) A biaxial single crystal will appear extinct when the vibration directions of the crystal are parallel to the crosshairs on the microscope, which are set to be parallel to the crossed polars. When the stage (and crystal) is rotated 45° , the crystal will become fully birefringent (brightly illuminated). (B) Retardation plates can be used to identify the optical properties of a crystal, such as slow and fast directions (which appear yellow and blue in this example). We use the gypsum 1st order red λ plate because it enables one to see both amorphous and crystalline materials. The gypsum plate produces an additional 550 nm of retardation, which changes the interference color of white light into magenta. Amorphous materials, or birefringent materials in the extinct position, will appear the same magenta color as the isotropic background. The outline of an amorphous material, which would be dark without the gypsum plate, can then be seen. (C) Spherulitic crystals display a Maltese cross pattern under crossed polars due to the radial orientation of the polycrystals. The Maltese cross remains stationary, even as the sample stage is rotated. When using the gypsum plate, the Maltese cross still arises from the extinct position of the radial crystals, but it will now appear magenta. In addition, the alternate sectors of the sphere will have opposite vibration directions from the opposing direction of the radial crystals, and therefore will display opposing retardation colors.

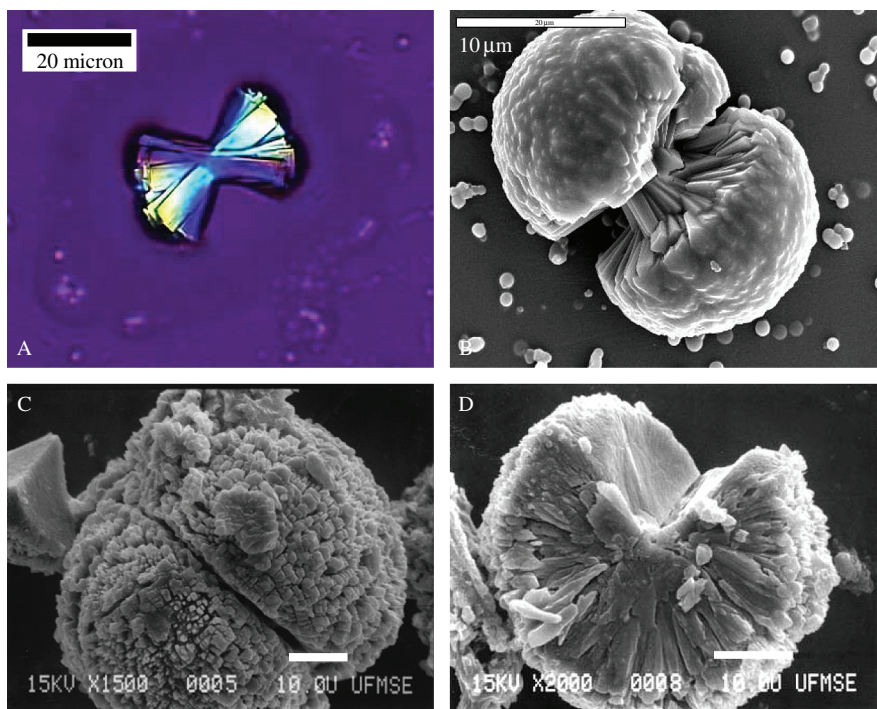


Plate 7 (Figure 4.39) Spherulitic morphologies of calcium oxalate. (A) Partial spherulite of 'sheaf of wheat' morphology. The shape of the individual polycrystals is flat platelets, which are most likely COM crystals. (B) Partial spherulite of 'dumbbell' morphology. The small round particles surrounding the sphere are 'droplets' of PILP phase. (C) The polycrystals have branched all the way around to form a double lobed spherulite. (D) Fracture surface of a full single lobed spherulite, showing the radial texture of the densely packed polycrystals.

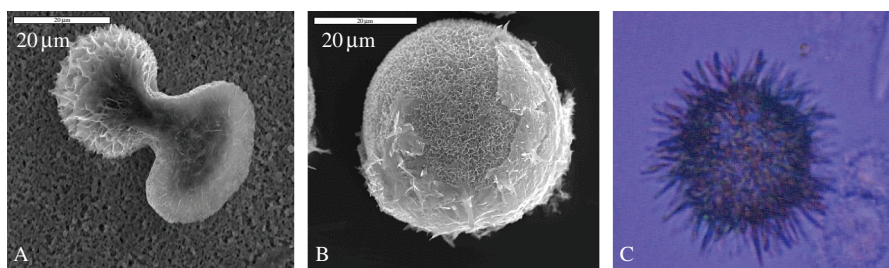


Plate 8 (Figure 4.40) Spherulitic morphologies of calcium phosphate. Note, platy clusters of HA can also grow from ACP, as shown in Figures 4.14 and 4.24A of the Bone section. (A) Partial spherulite of 'dumbbell' morphology. The shape of the individual polycrystals of the left bell appears to be irregular platelets, as is typical of the HA clusters mentioned above. (B) Full spherulite composed of densely packed polycrystals. The surface appears to be transforming into larger platelets. (C) 'Pin cushion' spherulite composed of needles (probably HA). These spherulites originated from smooth amorphous looking globules (such as at the bottom right), which apparently underwent surface dissolution and recrystallization into the more typical inorganic habit of needles.

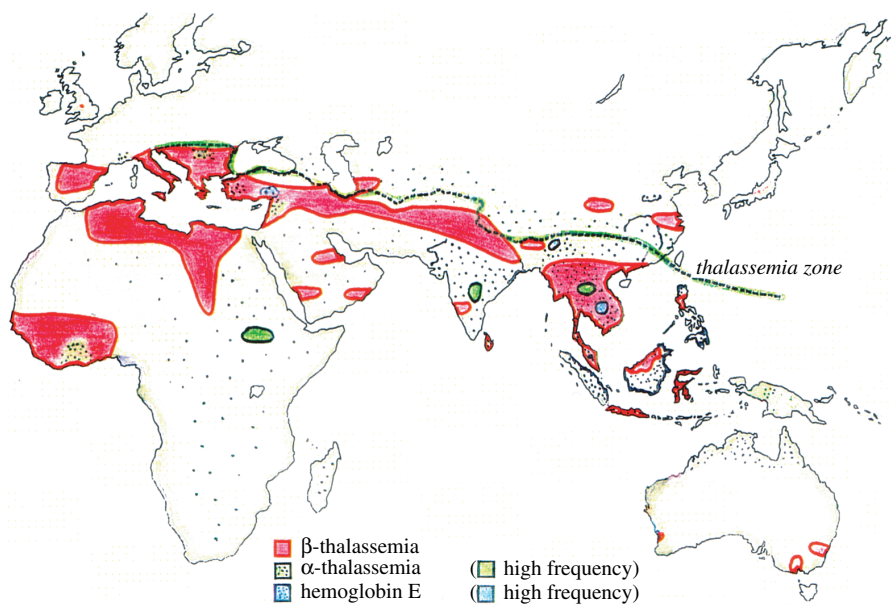


Plate 9 (Figure 5.1) Global distribution of thalassemic genes.

1 Solubility Phenomena Related to Normal and Pathological Biomineralization Processes

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1.1 INTRODUCTION

Biomineralization, which refers to the complex processes by which organisms form minerals, is frequently associated with a high degree of regulation on different hierarchical levels [1,2]. ‘Biologically controlled’ mineralization, in which extra-, inter- and intracellular activities direct the nucleation, growth and morphology of minerals that form ‘normal’ biomaterials such as bone and teeth [1,2], is fundamentally different from ‘biologically induced’ mineralization, which occurs as a result of interactions between biological activity (affecting e.g. the pH and composition of secretion products) and the environment [1,2]. Since there is little control of the biological system over the type and habit of minerals deposited, these vary as greatly as the environments in which they form and are often poorly defined, heterogeneous and porous [1,2]. Biologically induced mineralization is commonly associated with various bacterial activities and with epicellular mineralization in marine environments, occasionally leading to the complete encrustation of organisms, that sink subsequently and form sediments [1,2]. However, its characteristic features are also typical for uncontrolled ‘pathological’ crystallization resulting in painful or even life threatening conditions such as calculi formation (renal, biliary, pancreatic or sublingual), development of gout or arteriosclerosis, tissue calcification associated with cancer, etc.

In any event, solubility phenomena, i.e. dissolution and precipitation reactions, are fundamental to all biologically controlled or induced mineralization. Solubility phenomena in multicomponent electrolyte solutions also control numerous other ‘real-life’ natural or industrial processes. These include interactions in the hydrogeological cycle such as hydrothermal mineral formation, weathering and aerosol formation. The recent growth in biogeochemistry stresses the interrelations between biological and abiotic mineralization in the understanding of the past

and future evolution of the Earth. Solubility phenomena are furthermore relevant to procedures for preparing, separating and purifying chemicals and (biomimetic) materials industrially or in the laboratory. The study of solubility phenomena also serves to elucidate the mechanisms of unwanted precipitation and develop methods for its prevention, both industrially (scaling) and biologically (pathological mineralization), thus highlighting important analogies between two apparently distinct areas.

The kinetics of dissolution and precipitation has frequently been studied for both biological and industrial systems. The rate equations used to interpret such kinetic data are commonly defined in terms of under- and supersaturation respectively [3], which requires accurate solubility data for the solid substances at the pertinent conditions. However, in some studies, such as in a fundamental investigation of calcite growth kinetics [4], equilibrium solubilities have been derived from the very same rate data they ought to rationalize, which might lead to a correlation between these solubility values and the parameters of the rate model so obtained. It is thus imperative to employ the most accurate solubility data that are available from independent solubility studies, as reviewed for calcite [5].

For the calculation of biomineral solubilities in complex biological fluids, a suitable model for aqueous activity coefficients together with stability constants of the various complex species formed in the solution as well as the solubility constants of the solid phases are required. Therefore it is important to obtain reliable information about these constants from accurate physicochemical measurements. These methods, together with techniques to measure the kinetics of precipitation and dissolution, will be briefly outlined in the following section.

1.2 EXPERIMENTAL METHODS

1.2.1 SOLUBILITY MEASUREMENTS

The majority of normal and pathological biominerals formed in humans are sparingly soluble electrolytes with basic anions (i.e. anions that can be protonated), such as phosphates, carbonates, oxalates, urates, etc. Their solubility thus depends (often strongly) on pH and is in general decreasing as the pH increases. A notable exception is uric acid, whose solubility increases with pH.

Reliable techniques to measure solubilities of sparingly soluble electrolytes with basic anions were reviewed recently [6]. The recommended method consists of equilibrating the solid phase and the solution in thermostatted, all glass, percolation type solubility cells equipped with glass and reference electrodes [7–14]. The pH variation method (i.e. the systematic variation of the initial H^+ concentration from run to run) was used. Constant ionic strength media were employed throughout to keep the activity coefficients of the reacting species essentially constant. Thus, hydrogen ion concentrations (rather than activities) were measured potentiometrically and hereafter $p[H]$, defined as $p[H] = -\log\{[H^+]/\text{mol dm}^{-3}\}$

will frequently be used instead of pH. The metal ion and organic anion concentrations were determined by standard analytical methods, such as AAS and UV spectrophotometry respectively.

1.2.2 SOLUTION CALORIMETRY

The direct measurement of enthalpies of solution of solid phases provides important information on thermodynamic consistency by comparison with the enthalpy values derived from the temperature dependence of solubility constants. For instance, isoperibolic solution calorimeters were employed to measure the dissolution enthalpies of the calcium oxalate hydrates [12], uric acid anhydrate and dihydrate [15] and xanthine [14]. In the first case, a thermodynamic cycle was employed to obtain the dissolution enthalpy at ionic strength zero [12]. In the other two studies, the ionic strengths were adjusted to the values employed at the solubility measurements and TRIS buffer solutions of appropriate pH were used to increase the solubility and to ensure a defined final state of predominantly hydrogenurate and hydrogenxanthinate respectively [14,15]. In all cases, it was found that temperature-dependent solubility constants and calorimetrically measured enthalpies of solution were thermodynamically consistent [12,14,15].

Solution calorimetry has proved very useful for studying the energetics of iron oxide/oxyhydroxide and other nanoparticles [16]. Measurements have been performed either near room temperature (with an acid as the solvent) or at 700 to 800°C in an oxide melt. Both nano- and bulk materials of the same composition were reacted under the same conditions. The enthalpy difference between the two measurements is related to the difference in the surface energies of bulk and nanomaterial. Such differences are not only useful for the evaluation of the relationship between particle size and solubility, they may also serve to stabilize, in the nanoregime, polymorphs that are not stable in the bulk [16,17].

1.2.3 KINETIC MEASUREMENTS

Studies of the dissolution and crystallization kinetics of solids first require the preparation of metastable under- and supersaturated solutions, followed by appropriate measurement of the respective reaction rates. One of the earliest experimental approaches was that of 'free drift' in which the rates are obtained by measuring concentration changes as function of time [18,19]. To keep the thermodynamic driving forces (i.e. the activities of the reacting ions) constant, the 'constant composition' (CC) method pioneered by Nancollas [20] is widely used nowadays for the determination of dissolution and growth kinetics [21]. This technique can also mimic biomineralization processes during which constant ionic concentrations are regulated by homeostasis. To simulate, in addition, the slow crystallization rates

commonly prevailing *in vivo*, the CC method has been combined with a double-diffusion (DD) technique [22]. This ‘CCDD method’ has been applied to simulated body fluids resulting in the growth of carbonate apatites very similar to biological specimens [22].

As there are close relationships among the observed solubilities, the kinetics of dissolution and crystallization, and the interfacial tensions between the solid phases and their solutions, the accurate measurement of the latter has received considerable attention [21,23].

1.3 THERMODYNAMIC MODELING OF BIOLOGICAL SYSTEMS

1.3.1 INTRODUCTION

D.R. Williams [24] was one of the first who proposed and systematically pursued the idea that chemical equilibria in biological systems can be studied by the very same, well established experimental and computational methods that have been used in solution chemistry for a long time. Once the formation constants of all (or at least the most important) metal–ligand complexes have been characterised *in vitro* either experimentally (e.g. by potentiometric titration) or by appropriate estimation methods, the so-called speciation (i.e. the distribution of the metal among its low molecular weight complexes) can be calculated. This can e.g. be achieved by solving a system of equations derived from the law of mass action using suitable mass balance equations as a constraint. It is then assumed that the speciation established in this way reflects the metal–ligand distribution in the biological system *in vivo*, which in turn permits conclusions to be made about metal toxicity and bioavailability, metabolism, mobilization and immobilization, transport, deposition, etc. It has to be understood, however, that due to the complexity of some biological fluids containing a large number of N-, O- and S-ligands, the species distribution of a metal *in vivo* among the low-molecular-weight ligands such as amino and organic acids is never completely known. Nevertheless, this approach has proved successful for many applications, some of which are outlined below while others, related to bioinorganic chemistry, have been reviewed recently [25].

1.3.2 CHEMICAL SPECIATION, BLOOD PLASMA MODELS AND CHELATION THERAPY

Among the most prominent applications of (quasi)equilibrium calculations for biological systems are computer simulations of metal ion distributions amongst the low molecular weight ligands in blood plasma. These ‘blood plasma models’ were pioneered by Perrin [26] and were further developed in various laboratories, as reviewed by May [27]. The term quasiequilibrium indicates that the system of metal ions and organic ligands does not attain a stable thermodynamic equilibrium

state, which would imply, for instance, that the ligands decompose when conditions are oxidizing. One of the most sophisticated computer codes for this kind of simulations is the JESS (Joint Expert Speciation System) package of computer programs [28–31], which contains an extensive thermodynamic database. JESS can handle equilibrium calculations involving thousands of species and is also able to take redox equilibria and kinetic constraints [32] into account.

Data base improvements, e.g. due to the measurement of new complex formation constants that were not known before, have changed likely species distributions in blood plasma dramatically. For instance, older models have indicated that Fe(III) and Cu(II) complexes predominate, while modern computer simulations which include redox equilibria suggest that these two metals complexed by low molecular weight ligands are overwhelmingly present in blood plasma as Fe(II) and Cu(I) species [33,34 and references therein].

Due to the binding of metals to proteins, blood plasma models are unable to provide absolute species concentrations *in vivo*, however, they can give valuable information on the competitiveness of low molecular weight ligands for metal ions in solution, which is expressed as relative (percentage) species distribution. Thus, *trends* in such species distributions can be established when the homeostatically regulated metal or ligand concentrations become imbalanced. Disruptions of normal metal homeostasis may lead to conditions such as thalassemia or Wilson's disease [35–40] (Cu and Fe overload respectively, leading to the depositions of corresponding solids) or to Alzheimer's disease, with an associated deposition of solids in the brain, including an alleged Cu^{2+} induced aggregation of β -amyloid (A β plaques) [41] and nanoscale magnetic biominerals such as magnetite and maghemite [42]. These deposits result in Fe [43] and Cu [44] redox cycling [45], i.e. a metalloenzyme like activity [46], leading to oxidative stress by continuous H_2O_2 generation which probably accelerates the degeneration of brain tissue [47].

Metal overload in humans can be treated by administering chelating agents that help to excrete the excess metal. Effective drugs for chelation therapy have often been identified by computer simulations [48]. For instance, the treatment of Wilson's disease requires life long administration of an appropriate Cu chelator such as D-penicillamine, which was introduced half a century ago [49,50] and is still regarded as one of the most effective drugs [35,40], although alternatives have been recommended [51,52]. However, the study mentioned above [33], which used a newly determined set of formation constants for Cu(I) thioamino acid complexes, arrived at the conclusion that the mechanism of copper removal by penicillamine *in vivo* is unlikely to depend on complexation alone, in contrast to earlier simulations that apparently confirmed its therapeutic action [53]. Other Cu chelators have been shown to dissolve A β plaques *in vitro* [54] and in post-mortem brain tissue [55,56]. Recently, it has been suggested that D-penicillamine carried by nanoparticles (which had been found to be able to cross the blood–brain barrier) has the potential to prevent the A β accumulation in the brain observed in Alzheimer's disease [57]. The development of agents that can selectively prevent transition metals from binding to the A β peptide without perturbing the action of other metal containing biomolecules

in the brain [58] and therapies that focus on intervening in the roles of metal ions in oxidative stress [59] are currently of high priority.

1.3.3 METAL SOLUBILITY AND TOXICITY

As many metals are biotransformed in humans to a limited extent, it is often the speciation *before* entering the body that determines toxicity, as is, for instance, the case for Ni, As or Hg compounds [60]. While some metals are completely inert biologically (e.g. Ta [61]), solubility may be a criterion for the toxicological assessment of others (nontoxic, sparingly soluble BaSO_4 vs toxic, soluble BaCl_2 as opposed to slightly toxic, soluble and hence easily excretable NiCl_2 vs carcinogenic, sparingly soluble Ni_3S_2 which becomes phagocytosed in particulate form and consequently leads to very high intercellular Ni concentrations [62]). Moreover, it is often important to distinguish between organic and inorganic species of the metal, such as mercury [63]. Hg(II) undergoes bioalkylation in the environment and the resulting, highly toxic CH_3Hg^+ ion accumulates in fish and shellfish and is not metabolized further in the human body [64]. On the other hand, nontoxic arsenosugars may also be ingested from seafood and form a significant fraction of the total blood As in humans. However, exposure to toxic inorganic As is indicated by the occurrence of mono- and dimethylarsenates in blood, which are the major As metabolites in humans [65].

For other substances, speciation may change dramatically in the body, e.g. upon the passage from the stomach ($\text{pH} \approx 1\text{--}2$) to the intestine and subsequent absorption into the blood ($\text{pH} \approx 7.4$). Under the latter conditions, metals like Fe or Al form very slightly soluble hydroxides, however, Al absorption (and hence toxicity) can be greatly increased, due to complexation, by coingestion with citrate or tartrate, both of which are commonly found in fruits and in industrial foods and drinks [66]. It has also been reported that ulcer patients (with associated excess acid production in the stomach) had increased serum and urine Al levels on an Al hydroxide absorption test, which indicates a dependence of gastrointestinal Al hydroxide absorption on gastric pH and hence implies a potential risk of prolonged administration of antacids containing Al [67].

Sutton and Burastero have studied the chemical speciation and solubility of Be [68] and U(VI) [69] in various body fluids by computer simulation, however, only the inorganic ligands have been taken into account. The authors found that the results vary markedly between each biological fluid due to differences in (inorganic) fluid composition, ionic strength and pH. It turned out that Be and U(VI) phosphate solubilities control the metal concentrations in many of the biological fluids studied. It is noteworthy that phosphate solubilities apparently control lead concentrations in natural environments [70] and are inversely correlated to divalent metal accumulation in freshwater bivalves [71]. Although the authors [68,69] claim that their results aid in understanding the metabolism and toxic effects of Be and U(VI) and can potentially be applied to chelation treatment of chronic beryllium

disease [72] and uranium overexposure, the effect of organic ligands on speciation and solubility still needs to be assessed. Duffield [73] has developed an equilibrium model of plutonium in blood plasma which takes into account the roles of the iron transport protein transferrin (which binds most of the Pu) and of the Pu-citrate complex for the distribution and excretion of Pu in mammalian systems (citrate is often considered as a model system for more complicated naturally occurring proteins). A comprehensive review [74] also emphasizes the importance of both Pu and uranyl binding to transferrin in blood plasma.

The effect of the oxidation state on toxicity is well known for chromium: whilst Cr(III) is believed to be essential, Cr(VI) is carcinogenic. Mercury can be detoxified by mercury resistant bacteria either by precipitation as HgS, by biomineralization of Hg as an insoluble Hg-S complex other than HgS (probably due to the aerobic production of a volatile thiol compound) or by enzymatic reduction to Hg⁰, which is volatile and diffuses freely out of the cell [75]. These mechanisms of mercury detoxification can be utilised for Hg bioremediation from waste water [75]. It has also been hypothesised that uranium can be immobilized by a biomineralization process through precipitation of microbially produced phosphate and U(VI) [76]. The important role of humic acids for metal binding and their relation to biomineralization has also been emphasised [77].

1.3.4 URINE MODELS

Urolithiasis, i.e. the formation of stones or calculi in the urinary tract, is not only a painful condition affecting some 10 % of the population in industrialized countries but also causes annual costs to the health system in the order of millions of dollars estimated per 1000 patients that undergo treatment [78]. For decades, urolithiasis has arguably been the most research intensive sector of clinical and fundamental investigations into the cause, prevention and treatment of crystal deposition diseases in humans. However, it appears that a real breakthrough in this area is lacking as yet.

Human body fluids are normally supersaturated with regard to several substances (e.g. blood plasma, interstitial and intracellular liquors with respect to calcium carbonates and phosphates, particularly hydroxyapatite and fluoroapatite; bile with respect to cholesterol; urine with respect to calcium oxalates and, depending on the pH, with regard to uric acid or calcium phosphates). The question, why pathological crystallization does not occur indiscriminately in all humans, has been discussed in terms of three main factors: besides (i) the supersaturation as a necessary condition, (ii) the presence of heterogeneous nucleants and (iii) a deficit of crystallization inhibitors play a crucial role in pathological situations [79].

For the calculation of urinary saturation with respect to stone-forming substances, the usefulness of modeling the ionic equilibria in urine by computer simulations has been clearly demonstrated [80–94]. As mentioned above, these models use the stability constants of the various complex species formed in urine as well as the

solubility constants of the stone-forming solid phases and thus permit simulations that allow the judgement, from a physicochemical perspective, of various therapies of renal lithiasis suggested in the literature.

In a comprehensive review, more than 20 different types of renal stones have been classified [95] (see also Chapter 2 in this book). These stones or calculi are composed of calcium oxalate hydrates (hereafter COM for monohydrate and COD for dihydrate), ammonium magnesium phosphate (struvite), calcium phosphates (hydroxyapatite, HAP, and brushite, DCPD), uric acid and urates, cystine and xanthine. A sound knowledge of the solubilities of these substances is necessary to understand the cause, prevention and treatment of renal or bladder calculi. However, when the available experimentally determined solubility data of these substances were critically assessed they were found to be either sparse or in large disagreement [7]. Consequently, solubility measurements were performed in our laboratory at Leoben University (Austria) so as to provide reliable data for these compounds over wide ranges of experimental conditions, particularly those most pertinent to urolithiasis [8–14]. Special care was taken to demonstrate the consistency of these equilibrium constants with other thermodynamic, particularly calorimetric, quantities [12,14,15].

The solubilities of calcium oxalate hydrates were modeled using the JESS suite of computer programs [28–31] and the solubility constants ($\log K_{s0}$) determined in our laboratory, whereas in the case of calcium and magnesium phosphates literature values were used (see Chapter 3 in this book for a review). In these simulations, all possible complexes were considered whose formation constants were taken from the JESS thermodynamic database. Also, one of the in-built activity coefficient models of JESS was used (Davies equation). In the urine model developed by the authors [7,96], citrate and oxalate were considered besides the inorganic salts. Regarding the number of species (213), reactions (265) and thermodynamic quantities (more than 4000, including enthalpy, free energy and heat capacity values), this urine model was possibly the largest ever at that time.

Later [12], this urine model was extended significantly by a considerable increase in the number of species (280), reactions (380) and thermodynamic quantities (some 7200, mainly equilibrium constants but also standard potentials, Gibbs energies, enthalpies and heat capacities).

In the following sections, we present a discussion of solubility data and their application to the modeling of urinary saturation for all important components of renal calculi, except calcium and magnesium phosphates which are dealt with in Chapter 3 of this book. We start with uric acid, urates, cystine and xanthine, for which there are only a small number of equilibrium constants required to model the solubility in a great variety of salt solutions, including artificial urine, so that sophisticated simulation programs are not necessary.

Uric Acid and Urates

Uric acid ($C_5H_4N_4O_3$) is the major end product of the purine metabolism in humans. Uric acid stones may be idiopathic or secondary to a systemic disease such as

gout, which is induced by the deposition of needle shaped sodium hydrogenurate monohydrate crystals in joints [97]. While hyperuricosuria and low urinary output are well known contributing factors, the most important risk factor for uric acid stone formation is persistently acidic urine [98–101]. The solubilities of uric acid and its salts exhibit considerable dependencies on pH and temperature [9–12,15], see Figures 1.1 and 1.2. In the pH range of urine, three equilibrium constants are

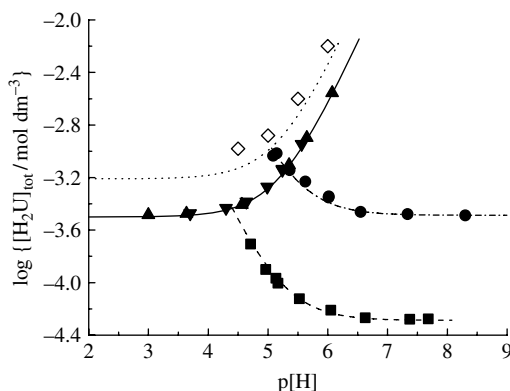


Figure 1.1 Solubility of uric acids and hydrogenurates at 37°C (adapted from [12]). Uric acid anhydrate: solid inverted triangles, in Standard Reference Artificial Urine [10]; solid triangles, in $0.300 \text{ mol dm}^{-3} \text{ NaCl} + 0.050 \text{ mol dm}^{-3} \text{ creatinine}$ [10]. Open diamonds [103] obviously correspond to uric acid dihydrate. Sodium hydrogenurate monohydrate: dots, in $0.150 \text{ mol dm}^{-3} \text{ NaCl}$ [9]; ammonium hydrogenurate: solid squares, in $0.300 \text{ mol dm}^{-3} \text{ NH}_4\text{Cl}$ [12]. The lines were calculated using the equilibrium constants given in Tables 1.1 and 1.2 (adapted from [12]).

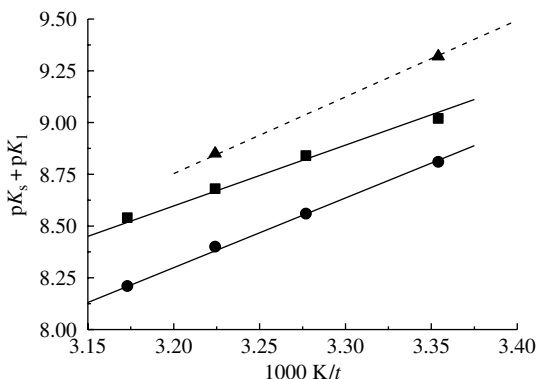
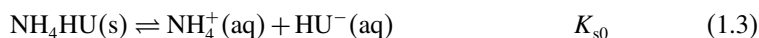
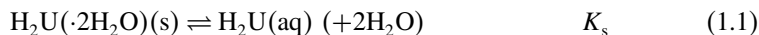


Figure 1.2 Consistency of thermodynamic data for xanthine (triangles), uric acid anhydrate (squares) and dihydrate (circles). Symbols are derived from solubility measurements; the slopes of the lines were obtained calorimetrically [14,15]. Data for xanthine were shifted by -2 units for better representation (adapted from [12]).

required to calculate the solubility. In Reactions (1.1–1.3), uric acid anhydrate, the metastable uric acid dihydrate and ammonium hydrogenurate were taken as examples:



Dissociation of the second proton of uric acid occurs at pH values far exceeding the physiologically important range. The solubilities of uric acid and ammonium hydrogenurate are given by Equations (1.4) and (1.5) respectively

$$[\text{U}]_{\text{tot}} = [\text{H}_2\text{U}] + [\text{HU}^-] = K_s(1 + K_1/[\text{H}^+]) \quad (1.4)$$

$$\begin{aligned} [\text{U}]_{\text{tot}} &= [\text{H}_2\text{U}] + [\text{HU}^-] = [\text{HU}^-][\text{H}^+]/K_1 + [\text{HU}^-] \\ &= [\text{HU}^-]([\text{H}^+]/K_1 + 1) \\ &= K_{s0}([\text{H}^+]/K_1 + 1)/[\text{NH}_4^+] \end{aligned} \quad (1.5)$$

Thus, the equilibrium constants of Reactions (1.1–1.3) can be calculated from least-squares analyses of solubility data.

For the modeling of uric acid and hydrogenurate solubilities, a thermodynamically consistent set of equilibrium constants and calorimetric data has been obtained in our laboratory (see Tables 1.1 and 1.2, Figures 1.1 and 1.2) [9,15]. Moreover our experimental results have proved that in the ionic strength range $0.15 \leq I_c/\text{mol dm}^{-3} \leq 0.30$, the solubility of uric acid neither depends on the nature and concentration of various inorganic components of urine nor on the presence of organic substances like urea and creatinine [10]. Thus, the same solubility as in the other salt solutions was also found [10] in so-called Standard Reference Artificial Urine, whose composition is given in [102].

Table 1.1 Solubility and first dissociation constants at 37 °C of xanthine (obtained from solubility measurements at $I_c = 0.300 \text{ mol dm}^{-3}$ NaCl [14]), uric acid anhydrate (H_2U) and dihydrate ($\text{H}_2\text{U} \cdot 2\text{H}_2\text{O}$), valid for various salt solutions and artificial urine in the ionic strength range from 0.15 to 0.30 mol dm^{-3} , as derived from solubility measurements [9,10]. The enthalpies of solution were measured calorimetrically and correspond to the reactions $\text{H}_2\text{Xan}(\text{s}) \rightarrow \text{H}^+(\text{aq}) + \text{HXan}^-(\text{aq})$ [11] and $\text{H}_2\text{U} \cdot (2\text{H}_2\text{O})(\text{s}) \rightarrow \text{H}^+(\text{aq}) + \text{HU}^-(\text{aq}) (+2\text{H}_2\text{O})$ respectively [15].

Substance	pK_s	pK_1	$\Delta_r H/\text{kJ mol}^{-1}$
Xanthine	3.69 ± 0.03	7.16 ± 0.03	71.0 ± 1.3
Uric acid anhydrate	3.49 ± 0.03	5.19 ± 0.04	56.3 ± 0.4
Uric acid dihydrate	3.21 ± 0.01		64.5 ± 0.2

Table 1.2 Solubility products of hydrogenurates at 37°C.

pK_{s0}^a [9] (NaHU · H ₂ O)	pK_{s0}^b [12] (NH ₄ HU)	pK_{s0}^c [108] (Ca(HU) ₂ · 6H ₂ O)	pK_{s0}^d [109] (KHU)	pK_{s0}^d [109] (LiHU · 1.5H ₂ O)
4.31 ± 0.01	4.80 ± 0.01	9.28 ± 0.04	3.85 ± 0.05	2.75 ± 0.05

^a $I_c = 0.150 \text{ mol dm}^{-3}$ NaCl, ^b $I_c = 0.300 \text{ mol dm}^{-3}$ NH₄Cl, ^c $I = 0$, ^d $I_c = 0.15 \text{ mol dm}^{-3}$ LiCl.

At 37°C, the solubility of the metastable uric acid dihydrate exceeds that of the anhydrate by a factor of about two. The solubility data obtained by Sperling and de Vries [103] obviously correspond to the dihydrate (Figure 1.1). In fact, the uric acid samples of [103] were precipitated by acidification of real urine but were not characterized. It has been reported that under these conditions of high supersaturation, uric acid dihydrate is formed [104].

Urinary alkalization with potassium citrate or sodium bicarbonate is a highly effective treatment, resulting in dissolution of existing stones and prevention of recurrence [100]. Excessive increase of the urinary pH, however, may cause precipitation of sodium or ammonium hydrogenurates [105]. The latter substance has also been found together with struvite in infectious stones caused by urea-splitting bacteria. It has been reported that *in vitro*, uric acid stones dissolve better in lithium carbonate than in sodium or potassium (hydrogen)carbonate solutions; this behaviour was attributed to a litholytic effect of lithium ions [106]. Our measurements have shown, however, that uric acid has the same solubility in lithium and sodium chloride solutions [11]. The increased solubility of uric acid in lithium carbonate solutions is obviously due to a higher pH and to the fact that lithium hydrogenurate has a higher solubility than the corresponding sodium and potassium salts (Table 1.2). The latter compounds may form sparingly soluble precipitates on the surface of the uric acid calculus and prevent further dissolution even if the pH is increased (see Figure 1.1).

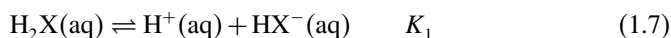
Sodium hydrogenurate monohydrate, whose crystallization in synovial fluids around joints is the first step of gouty inflammation, has its lowest solubility at physiological pH (Figure 1.1). One of the mysteries of gout is that only a small percentage of individuals with hyperuricaemic body fluids (which are supersaturated with respect to sodium hydrogenurate monohydrate) have ever had a gouty attack. This may be related to the fact that this substance can form solutions that are highly supersaturated without any crystallization occurring, a result also found in our solubility study [9]. Whereas synovial fluids of gouty patients have nucleated sodium hydrogenurate monohydrate, normal synovial fluids, serum albumin and heparin inhibit its crystallization [107].

Xanthine

Xanthine (C₅H₄N₄O₂) is the intermediate product of the purine metabolism in humans and is metabolized to the final product uric acid (C₅H₄N₄O₃) by xanthine

dehydrogenase (XDH). Classical xanthinuria, a very rare condition first described in 1954 [110], is classified into two categories: type I, deficient only in XDH activity; and type II, deficient in both XDH and aldehyde oxidase. Both types are associated mainly with renal stones and lead to renal failure in some cases [111]. Treatment of gout with allopurinol (which inhibits XDH) has been reported as another cause of xanthine stones [112].

Similar to uric acid, the solubility of xanthine exhibits significant dependencies on pH and temperature [14], see Figures 1.2 and 1.3. In the pH range of urine, two equilibrium constants are required to calculate the solubility.



Reactions (1.6) and (1.7) are analogous to anhydrous uric acid, thus the solubility can be described by an expression analogous to Equation (1.4). Solubility products of sparingly soluble hydrogenxanthinates, analogous to Reaction (1.3), have not been reported. In both cases (uric acid and xanthine), dissociation of the second proton occurs at p[H] values far exceeding the physiologically important range. Similar to uric acid, the equilibrium constants of Reactions (1.6) and (1.7) can be calculated from least squares analyses of solubility data.

The thermodynamic quantities describing the solubility of xanthine, as obtained in our laboratory [14], are presented in Table 1.1, Figures 1.2 and 1.3. The enthalpy of solution calculated from the experimentally determined solubility and first dissociation constants is $\Delta_r H = 67.9 \text{ kJ mol}^{-1}$. This value is in excellent agreement with the calorimetric value $\Delta_r H = (71.0 \pm 1.3) \text{ kJ mol}^{-1}$ [14]. Figure 1.2 reflects very well the thermodynamic consistency of our experimentally determined data obtained from two different methods, solubility and calorimetry. For comparison, literature data [113] are also shown in Figure 1.3. Lister and Caldbeck [113] reported

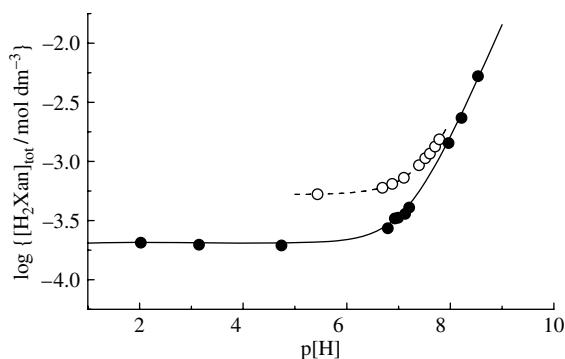


Figure 1.3 Solubility of xanthine at 37°C (adapted from [12]). Solid circles, [14]; open circles, [113]. The solid line was calculated using the equilibrium constants given in Table 1.1 (adapted from [12]).

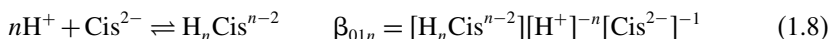
the solubility data of xanthine in some buffer solutions from which we obtained the solubility constants and the first dissociation constants given in [7,12,14]. The enthalpy of solution calculated from these data is $\Delta_r H = 22.9 \text{ kJ mol}^{-1}$ which differs significantly from our calorimetric value [14]. The experimental technique applied, which led to a considerably higher solubility, and the unreasonable decrease of the deprotonation constants with temperature indicate that the authors of [113] might actually have investigated supersaturated solutions.

Both K_s and K_1 of xanthine are lower than the corresponding values for uric acid. This means that xanthine has a lower solubility and litholysis by urinary alkalisation can become effective at a higher pH (by ca. 2 units) than in the case of uric acid. While some authors have nevertheless found beneficial effects of citrate in the prevention of xanthinuria [114], others opt for a high fluid, low purine intake as the only possible therapy for XDH deficiency [115].

Cystine

L-cystine, $\text{C}_6\text{H}_{12}\text{N}_2\text{O}_4\text{S}_2$, the least soluble of the naturally occurring amino acids, is normally excreted in urine in low concentrations of ca. $0.06\text{--}0.17 \text{ mmol dm}^{-3}$. Owing to a congenital defect in the tubular reabsorption of cystine, a small number of individuals excrete much higher concentrations of ca. $1.3\text{--}3.3 \text{ mmol dm}^{-3}$ which results in the formation of calculi that can block the renal tubes [116]. At least three cystinuria subtypes have been recognized and urine samples were compared to calculated solubilities in a recent study on cystinuria subtype classification [117].

The cystinate ion, Cis^{2-} , can be protonated in four steps according to



In Reaction (1.8), $n = 1, 2, 3$ or 4 , and β_{01n} denotes the corresponding protonation constants. The formally uncharged species $\text{H}_2\text{Cis}^\pm$ (which is actually a zwitterion) has the lowest solubility, i.e.,



and the corresponding (intrinsic) solubility constant is denoted as K_s . Thus, the solubility of cystine can be calculated as analytical function of $p[\text{H}]$ using five equilibrium constants (Table 1.3, Figure 1.4, see [13]). Since the ionic species are much more soluble, the total solubility of cystine is given by (Equation 1.10)

$$[\text{H}_2\text{Cis}]_{\text{tot}} = K_s(1 + (\beta_{012}[\text{H}^+]^2)^{-1} + \beta_{011}(\beta_{012}[\text{H}^+])^{-1} + \beta_{013}[\text{H}^+](\beta_{012})^{-1} + \beta_{014}[\text{H}^+]^2(\beta_{012})^{-1}) \quad (1.10)$$

Although the protonation constants of cystine were measured at $I_c = 0.15 \text{ mol dm}^{-3}$ [118], they reproduced solubility data measured at $I_c = 0.30 \text{ mol dm}^{-3}$ very well.

The solubility of cystine in oxalate free artificial urine was the same as in 0.30 mol dm^{-3} NaCl [13]. However, owing to the precipitation of phosphates from artificial urine at higher $p[H]$, data were only collected at $p[H] < 5.0$, while in phosphate free artificial urine, cystine solubilities were measured up to $p[H] = 8.2$. In the latter, a slightly higher solubility constant ($0.88 \text{ mmol dm}^{-3}$) was found, which is most likely due to complex formation of cystine with Ca^{2+} and Mg^{2+} , as was also confirmed by computer simulations with JESS [13]. In normal artificial urine, on the other hand, alkaline earth ions are complexed by phosphate. However, a significant dependence of the intrinsic solubility on the nature and concentration of various inorganic salts was reported in [119,120]; so more experimental work on this topic is certainly needed. Recent literature data for 0.5 mol dm^{-3} NaCl [121] agree with our values at low $p[H]$ but show a systematic deviation at high $p[H]$ [12]. It should be emphasised again that the excellent agreement between our measured solubility data and values calculated with independently determined protonation constants supports the reliability of both data sets.

Cystine solubilities in real urine [122] agree well with our results obtained in synthetic solutions [13] (Figure 1.4). Therefore, the equilibrium constants in Table 1.3 permit reasonable cystine solubility estimates for urine. In the formation of

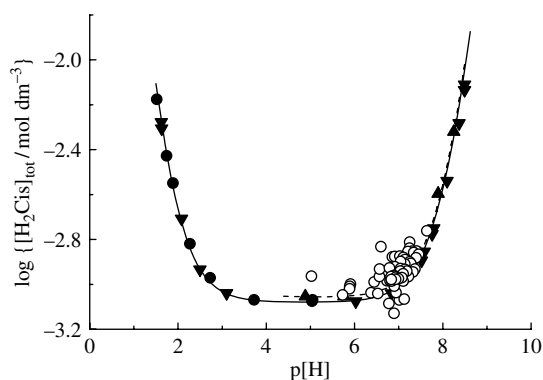


Figure 1.4 Solubility of L-cystine at 37°C (adapted from [12]): solid circles, oxalate-free artificial urine; triangles, phosphate and oxalate free artificial urine; inverted triangles, 0.30 mol dm^{-3} NaCl [13]; open circles, real urine [122]; solid line, calculated using equilibrium constants in Table 1.3; dashed line, calculated with JESS for phosphate- and oxalate free artificial urine [13] (adapted from [12]).

Table 1.3 Solubility and protonation constants for L-cystine used for solubility simulations in 0.30 mol dm^{-3} NaCl and oxalate free artificial urine at 37°C .

$-\log K_s^a$	$\log \beta_{011}^b$	$\log \beta_{012}^b$	$\log \beta_{013}^b$	$\log \beta_{014}^b$
3.08 ± 0.01	8.604 ± 0.003	16.356 ± 0.004	18.41 ± 0.01	20.03 ± 0.02

^a $I_c = 0.30 \text{ mol dm}^{-3}$ NaCl [13], ^b $t = 37^\circ\text{C}$, $I_c = 0.15 \text{ mol dm}^{-3}$ NaCl [118].

cystine stones, supersaturation is overwhelmingly important in relation to inhibition of crystallization and other factors (diet, infections, etc.) that may be relevant for other types of stone [123].

For the treatment of cystine lithiasis, potassium citrate has been used to increase the urinary pH and thus the cystine solubility [123]. However, a higher pH favours calcium phosphate calculi formation in stone prone patients. This problem would be particularly serious if a recent recommendation [124] to use THAM (tris-(hydroxymethylene)-aminomethane) buffer at pH = 10 for *in vivo* cystine chemolysis were applied. Even at pH $\approx$ 7, significant HAP precipitation is not only predicted by computer simulations [96] but also observed experimentally [96,125].

Calcium Oxalates

In contrast to the substances discussed above, the solubility of COM, the major component of oxalate calculi, is almost p[H] independent in the urinary p[H] range, as was shown by computer simulations and confirmed experimentally [8]. However, the calcium oxalate solubility strongly depends on the concentration of ions that form complexes with calcium or oxalate, particularly citrate or magnesium ions respectively [7,89]. It was demonstrated that our urine model [7,8] permits reliable solubility calculations by taking all of these complexes into account.

Owing to its importance for renal lithiasis, the solubility products of COM, COD and COT (calcium oxalate trihydrate) have been determined frequently. Nevertheless, the reliability of some of the early literature data is rather unsatisfactory. To clarify this point, a simple thermodynamic consistency test is applied according to a rule established almost 130 years ago [126]. This rule states that the enthalpies of dissolution become progressively more endothermic with extent of hydration, since the enthalpies of dehydration, corresponding to e.g. $\text{COT} \rightarrow \text{COD} + \text{H}_2\text{O}(\text{aq})$ or $\text{COD} \rightarrow \text{COM} + \text{H}_2\text{O}(\text{aq})$ are always positive [127]. The enthalpies of solution obtained from our solubility products (Table 1.4) obey this rule (see Figure 1.5) while those derived from some literature data, e.g. [128,129], do not [12]. Moreover the temperature dependence of our solubility products is consistent with values determined calorimetrically; details of these measurements

Table 1.4 Solubility products of the three calcium oxalate hydrates valid for $I = 0$ [8].

$t/^{\circ}\text{C}$	$-\log K_{s0}$		
	COM	COD	COT
20	8.84 ± 0.02	8.42 ± 0.02	8.33 ± 0.01
25	8.77 ± 0.01	8.34 ± 0.02	8.24 ± 0.01
30	8.71 ± 0.01	8.26 ± 0.03	8.12 ± 0.02
37	8.65 ± 0.03	8.17 ± 0.03	8.02 ± 0.02
40	8.62 ± 0.02	8.13 ± 0.04	7.97 ± 0.02

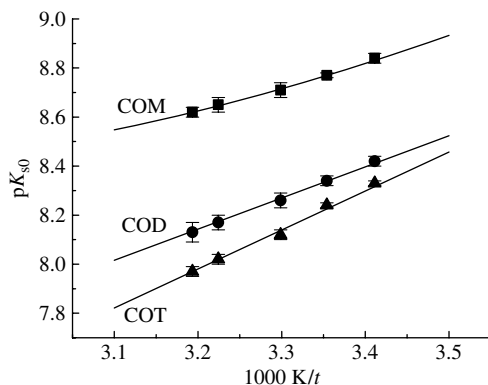


Figure 1.5 Thermodynamic consistency of calcium oxalate data (adapted from [12]). Solid symbols [8]; squares, COM; circles, COD; triangles, COT (Table 1.4). Solid lines correspond to calorimetrically determined enthalpies of solution [12] (adapted from [12]).

and the associated calculations are given elsewhere [12]. It should also be noted here that our thermodynamic quantities for uric acid anhydrate and dihydrate pass this consistency test as well (see Table 1.1 and Figure 1.2).

Urolithiasis is controlled by thermodynamic and kinetic factors either alone or in combination. At least for calcium oxalate and phosphate, the crystallization potential of urine is related not only to the concentration of any particular compound but also to the presence or absence of others, such as complexing agents, inhibitors or promoters of the crystallization of the compound in question. Crystallization (i.e. nucleation and/or crystal growth) inhibitors frequently cause the actual concentration products to exceed the corresponding solubility products. In this way, urine is supersaturated (metastable) with respect to some substances and kinetic factors play an important role to prevent or delay the precipitation of the substances. As supersaturation increases, a threshold may be reached at which urine can hold no more salt in solution and kinetic factors are no longer effective.

In a recent application of computer modeling to urolithiasis research, a correlation between COM, DCPD and HAP supersaturation in real urine samples and the results of a simple clinical test for urinary lithogen risk (ULR) was reported [130]. This correlation leads to an establishment of kinetic and thermodynamic factors contributing to stone formation. The ULR test results indicate the positive or negative risk of urinary calcium stone formation and the absorbance $A = 0.3$ has been established as the borderline between normal and lithogenic urines [131]. In Figure 1.6, results of this test are plotted vs the supersaturation of COM obtained from the urines of stone formers and healthy people. It can be seen that almost all human urines are supersaturated with respect to COM and as expected, urines of most healthy people give negative ULR test results. Six samples from this group give positive results and their log S values were employed to define a region called

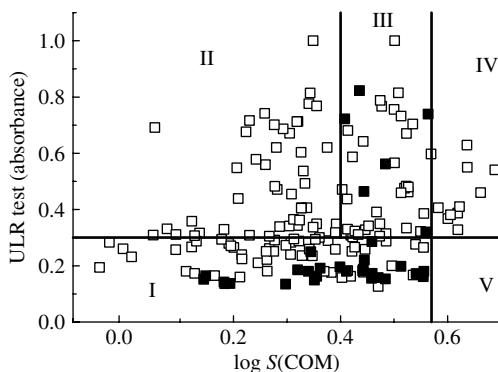


Figure 1.6 Correlation between ULR test and COM supersaturation (adapted from [12,130], with kind permission of Springer Science and Business Media). Open symbols, stone formers; solid symbols, healthy people.

‘kinetic threshold’ which was found to be $0.40 < \log S < 0.57$ for the crystallization of COM. Five cases corresponding to kinetic and/or thermodynamic control of urinary stone formation were established and they are presented as regions I, II, III, IV and V in Figure 1.6.

- (i) In region I, $0 < \log S < 0.57$ and $A < 0.3$ mean that the kinetic factors effectively keep calcium ions in solution. The presence of most of the data obtained from healthy people implies the absence of heterogeneous nucleants and/or the presence of some natural crystallization inhibitors in urine. The existence of about 1/3 of the data obtained from the stone-formers indicates that although having normal urine, these patients suffer some abnormal renal morphoanatomy or strongly disordered urodynamic conditions [131].
- (ii) In region II, $0 < \log S < 0.40$ and $A > 0.3$, COM crystallized from all urine samples. Data from stone formers are found exclusively which indicates the absence of crystallization inhibitors in their urines as well as abnormal morphoanatomy of their kidneys.
- (iii) Region III or the kinetic threshold consists of data with $0.40 < \log S < 0.57$ and $A > 0.3$. The width of this region depends on the uncertainties involved in the analyses of the samples. Six lithogenic samples from the healthy group found in this region signal that these people have excellent renal morphology so no stones were formed in their urinary tracts though the salt crystallized in the ULR test due to the high supersaturation [131].
- (iv) In region IV, $\log S > 0.57$ and $A > 0.3$, the supersaturation values are abnormally high so that thermodynamic factors are in absolute control and the only result is the crystallization of calcium oxalate. Obviously, kinetic factors do not have any effects even if there were inhibitors present in the tested urines. This is confirmed by the fact that only data from active stone formers with

positive ULR test results are found in this region. Consequently, for urines belonging to this region, it would be necessary to decrease the supersaturation prior to the administration of crystallization inhibitors, such as phytic acid, that are used as a pharmacological treatment of stone disease [79].

- (v) Region V ($\log S > 0.57$ and $A < 0.3$) does not contain any data since inhibitors are no longer effective at such high supersaturation.

In two recent studies on crystallization inhibition by urine, it has been found that male urinary calcium stone-formers lack proper inhibition of both nucleation and crystal growth [132] while women display only the former [133]. These and other studies use the 'upper limit of metastability' (ULM, i.e. the degree of supersaturation at which crystallization starts) as a measure of the threshold of nucleation and claim a (hitherto unexplained) correlation of ULM with supersaturation [134]. This is somewhat in contrast to the results shown in the ULR test/ $\log S$ plots above, in which urines with the highest supersaturation do not exhibit any kinetic inhibition of crystallization (in the ULR test – see Region IV). Nevertheless, at about $5 < S < 10$, the ULM/ S plots [132,133] display a region analogous to the 'kinetic threshold' (Region III) which contains urines of healthy people, whereas at higher S only urines of patients are found.

The effect of dietary factors and health supplements on the calculated supersaturation is of similar complexity [93,135]. Urinary oxalate has emerged as the most important determinant of calcium oxalate crystallization so that dietary oxalate intake should be restricted, while the role of urinary calcium has shifted to bone balance and osteoporosis. Dietary calcium restriction increases urinary oxalate and contributes to a negative bone balance and has therefore been abandoned as a means to reduce the risk of calcium oxalate kidney stone formation [135]. While it has been demonstrated that a sodium citrate containing preparation favorably alters the risk factors for calcium oxalate urolithiasis [136], the interplay between alkali, magnesium and citrate is complicated, given that the last two also inhibit the crystallization of Ca minerals [91]. Either synergistic effects (MgO and citrate on lowering the supersaturation of brushite and on increasing the pH) or additive effects (CaCO_3 and citrate on lowering the supersaturation of uric acid) have been proposed [137]. Data from various investigations on the effect of vitamin C are contradictory, in part because of difficulties regarding oxalate assay techniques [138–140]. Whereas cola consumption [141] has an adverse effect on calcium oxalate supersaturation, cranberry juice has antilithogenic properties [142]. The effect of increasing dietary oxalate on urinary calcium oxalate supersaturation is difficult to predict, as there may be increased absorption due to secondary hyperoxaluria [94], or oxalate may bind to intestinal calcium, which lowers calcium absorption and excretion [143]. Although black South Africans have higher calcium oxalate supersaturations than the white population, they seem to be immune to kidney stones [144]. Also, on an oxalate rich diet, their urinary oxalate did not increase markedly so that it has been proposed that lower oxalate absorption rates may be the reason that South African blacks hardly form stones [144].

As an important aspect of these models, it has recently been shown that urinary calcium is as equally effective as oxalate in increasing calcium oxalate supersaturation, when a widely accepted value [145] for the calcium oxalate stability constant is used [146]. As discussed in [146], this is in contrast to earlier studies, which had used a higher value for this constant and found that the influence of calcium on calcium oxalate supersaturation was less pronounced than that of oxalate.

A frequent recommendation to calcium oxalate stone-formers is to consume a large quantity of liquid [147]. Our computer simulations have demonstrated that this advice is physicochemically meaningful for certain fluids [12]. For pure water, only an unrealistically high 1:10 dilution results in an unsaturated solution, so only a high water load seems to effect a significant reduction of the supersaturation [148]. More likely, it mainly contributes to a faster flow of urine through kidney cavities and thus a removal of sediments. However, mineral waters containing complexing agents like magnesium can certainly reduce the supersaturation to a higher extent [12]. It was also concluded from the results of a study on 85 human subjects [149] that such mineral waters reduce the risk of calcium oxalate stone formation.

The Hypothesis of Urine as a Saturated Solution

Györy and Ashby proposed a new hypothesis about the thermodynamic state of urine, regarding it as a *saturated* solution with respect to stone forming substances [150–152]. This approach would imply that urine is at equilibrium with these solid, stone forming substances once their solubilities are exceeded. Györy and Ashby suggest that this suspension (or ‘quasicolloid’ [151]) is stable in normal individuals because of agglomeration inhibition (mainly by citrate) and base their hypothesis upon clinical observations, particularly crystalluria (the excretion of crystals in urine), and several modeling results. Kavanagh [153] has critically analyzed the arguments for and against the Györy and Ashby hypothesis and suggested how the authors could easily prove their hypothesis by ultrafiltration. However, no decisive experimental confirmation appears to have been carried out as yet, although no significant amount of nonultrafilterable calcium oxalate has been found in preliminary experiments [153]. While on the one hand recent research indicates that suspensions of very fine particles can be dynamically stabilized without undergoing dissolution in undersaturated supporting media [154], there is, on the other hand, direct evidence of supersaturation by seeded crystal growth experiments in urine. The formation mechanism of certain renal calculi also suggests that the Györy and Ashby hypothesis is questionable. For instance, columnar growth of papillary calcium oxalate monohydrate calculi (see Chapter 2 in this book) can be explained by supersaturation but hardly by agglomeration of colloidal crystals. Other apparent inconsistencies between urinary supersaturation and observed behaviour as claimed by Györy and Ashby (‘unsuccessful’ treatment by increasing the urine volume – see above; prediction of infection stones mixed with calcium oxalate) can be resolved by physicochemical considerations [153].

Nanobacteria

Recently, so-called nanobacteria, which behave like an extremely small microbe with very slow multiplication rate, have been found in kidney stones [155] and claimed to induce calcifications in various body fluids, including urine, by an apparent extraction and concentration of phosphate [156]. Nanobacteria appear to be correlated with such diverse conditions as arterial heart disease, Alzheimer's disease, malignant tumours and urolithiasis, which has recently been found to be worsened in astronauts during long space flights due to their seemingly enhanced growth in microgravity [157]. Although it is still controversial whether nanobacteria are living organisms (as their putative DNA has not been sequenced as yet [158]), it has been shown that various chemotherapeutics, including aminoglycoside antibiotics, inhibit the growth of putative nanobacteria *in vitro* and thus may help people who suffer from chronic stone formation and other conditions [159]. However, it has been suggested that these compounds might inhibit calcification rather than bacterial growth since biomineralization attributed to putative nanobacteria may be initiated by nonliving macromolecules including phospholipids and can be continued on dilution to fresh medium by microcrystalline apatite, which also accounts for the wide range of morphological forms ascribed to nanobacteria [158].

1.3.5 MODELING PANCREATIC AND BILIARY STONE FORMATION

Calcium carbonate is a major constituent of pancreatic stones (consisting of ca. 95 % calcite), salivary stones and many pigment gallstones, since these three gastrointestinal secretory organs generate high hydrogencarbonate concentrations and high pH values in their respective secretions. Moore and Verine have developed a physicochemical model of calcite saturation in pancreatic juice [160] that takes the solubility constant as well as the complexation constants of calcium ions with (hydrogen)carbonate and proteins into account. The simulations have shown that all of these ligands are important buffers for calcium ions in the juice [160]. Various *in vitro* studies have been performed to investigate the dissolution of stones as well as the influence of additives such as citric acid and dimethadione [161] or the effect of hormonal stimulation [162] on calcium carbonate saturation in pancreatic juice.

In industrialized societies, cholesterol rich gallstones are the most common type of stone found in the gall bladder [163]. As opposed to pigment stones that may contain bilirubin and are often associated with infections and hepatic cirrhosis, these stones are frequently composed of cholesterol microcrystals, where large specimens tend to form an outer shell which, among other calcium salts, contains vaterite, the least stable of the three crystalline anhydrous calcium carbonate modifications [164]. The complicated interplay of biliary cholesterol hypersecretion and hyposecretion of bile acids and phospholipids, which, among numerous other factors, may lead to supersaturation and nucleation of cholesterol microcrystals, has been discussed in a comprehensive review on the pathogenesis of gallstones [163]. Similar to urine, which is generally supersaturated with calcium oxalate, bile appears to be generally

supersaturated with cholesterol [163,165] so that kinetic factors determine whether or not stone formation occurs. A thermodynamic model of unconjugated bilirubin saturation in bile and a corresponding lithogenic index that discriminates lithogenic and non-lithogenic bile have been reported recently [166]. Chapter 3 in this book reviews further perspectives on the formation of gallstones and the development of atherosclerotic lesions, which also contain cholesterol and calcium salts [167,168]. Various aspects of salivary stones (sialoliths) and their formation mechanisms are discussed in Chapters 2 and 3 of this book.

1.4 NEW INSIGHTS IN SOLUBILITY PHENOMENA RELEVANT TO BIOMINERALIZATION

Modern experimental techniques have stimulated current research leading to a better understanding of the crucial role of solubility phenomena in biomineralization. Various new mechanisms that are potentially significant to normal and pathological biomineral formation have been proposed, such as mineralization of calcium salts via an amorphous liquid phase precursor [169]. This and other contemporary research, concerning the solubility of nanomaterials [154] and new studies on crystallization kinetics [170,171] are briefly outlined below.

1.4.1 SOLUBILITY OF NANOMATERIALS AND BIOLOGICAL DEMINERALIZATION

Biological materials frequently have nanosized mineral particles as their basic building blocks, for example, ferrihydrite in the iron storage protein ferritin [172] (further discussed in Chapter 5 of this book), magnetite in bacteria [1] or hydroxyapatite particles in bone, dentin and dental enamel [173,174]. As a very large fraction of the nanoparticle's atoms are near the surface, the effects of interfacial energy between particle and vacuum, a gaseous atmosphere, water or an aqueous solution become significant. The thermochemistry of nanomaterials has been reviewed by Navrotsky [16,17]. One of the most remarkable results of these studies is the thermodynamic stability of nanosized polymorphs that are metastable in the bulk. A general rule, demonstrated for many systems, states that metastable polymorphs (e.g. anatase) have lower interfacial energies than the stable phase (rutile), which results in a free energy crossover at high surface areas [16,17]. A similar behaviour has been found for nanocrystalline substances precipitating from aqueous solution, for instance, anatase with a crystallite size of 10–30 nm is formed first and recrystallizes on hydrothermal treatment to stable, much coarser rutile [175].

It has been established that there is a close relationship between solubility and interfacial energy [23,176,177]. During dissolution, ions on the surface are replaced by water molecules to escape into the bulk solution. As higher interfacial

energies indicate a greater difficulty in forming such an interface between the solid and aqueous phases, sparingly soluble, i.e. more stable, salts always have higher interfacial free energies than soluble salts. For example, it is well known that the interfacial energies of various calcium phosphate phases important in biomineralization increase in the order brushite < octacalcium phosphate (OCP) < β -tricalcium phosphate < hydroxyapatite < fluoroapatite whilst their solubilities decrease in the same order [23,177–179].

The general rule that metastable phases have lower interfacial energies and hence precipitate more easily than stable phases therefore also applies to aqueous systems. In addition, the ability of a surface to nucleate other phases is closely related to the magnitude of the interfacial energies [180]. For instance, it has been shown that calcium phosphates nucleate more readily on anatase than on rutile surfaces, owing to the lower interfacial energy of the former which favours the adhesion of the aqueous solution and thus facilitates the nucleation of calcium phosphate phases [181]. These relationships may also serve to explain Ostwald's law of stages, since the least stable phase tends to precipitate first and is able to nucleate more stable phases due to its lower interfacial energy. Therefore, the more soluble phosphates brushite and OCP are considered to be precursors for hydroxyapatite [178], as is amorphous calcium carbonate for crystalline calcium carbonate phases [182].

These examples indicate the importance of interfacial energies for rationalizing the dissolution and precipitation behaviour of sparingly soluble phases such as biominerals. However, the accurate measurement of this quantity has proved to be difficult since different techniques (solubility/particle size, nucleation, crystallization and dissolution kinetics, contact angle or wetting methods) yield vastly differing values for interfacial energies between solid and aqueous solution [21,23]. One research group has reported a positive [170] and a negative [178] value for the interfacial tension between brushite surfaces and water or solution (not clearly specified), although both values were measured using a thin layer wicking capillary rise technique. It should be noted that this technique is known to provide lower values than those obtained by other methods because the double-layer effects are also included [170].

Biomaterials like bone and teeth are examples of organic–inorganic nanocomposites that possess superior mechanical strength, as they are both hard and tough (fracture resistant) [183]. It seems that the weakening effect of flaws vanishes specifically at the nanoscale so that the strength of a perfect crystal is maintained despite defects [183]. The nanoscale dimension of the mineral phase of bone is also crucial to its bioresorptive potential and to the preparation of bone-graft substitutes (see Chapter 4 of this book). Moreover, recent dissolution studies of sparingly soluble Ca phosphates have revealed an unusual behaviour when the crystals fall under a critical size, also at the nanoscale, resulting in a kinetic self preservation of biominerals at undersaturation that prevents them from being dissolved [184].

Modern experimental techniques such as vertical scanning interferometry and *in situ* atomic force microscopy (AFM), which allow one to directly observe the dissolution behaviour of these biominerals, their synthetic analogs and

other minerals, have sparked a renewed interest in the theory of dissolution [154,174,184–191]. In traditional theories of dissolution, the dissolution rate has been expressed as a simple function of the relative undersaturation, which implies that the dissolution rate should remain constant at sustained undersaturation. However, constant composition dissolution studies of nanosized, synthetic brushite (calcium hydrogenphosphate dihydrate) [154,187], synthetic hydroxyapatite [186] and hydroxyapatite particles obtained from dental enamel [174,184] have shown that the dissolution rates decrease and become effectively suppressed even though the solutions remain undersaturated. This interesting and unusual behaviour has been explained in terms of a model that incorporates particle size considerations. It has been confirmed experimentally that demineralization of sparingly soluble phases is initiated and accompanied by the formation and development of pits on the crystal surfaces and that the dissolution rates are determined by the pit densities and spreading velocities [184–187]. It has been shown [174,184,185,192] that the dissolution rate depends on a critical pit size r^* and only pits of a radius r larger than r^* provide active sites that contribute to the dissolution,

$$R(r) \approx R_\infty(1 - r^*/r) \quad (1.11)$$

where R_∞ is the velocity of dissolution steps at $r \rightarrow \infty$. When $r \rightarrow r^*$, there is no fast movement of its stepwave, and the dissolution rate approaches zero. When the dimensions of the crystallites fall in the same order as r^* during the dissolution, the formation of active pits is more difficult due to size restrictions, leading to retarded dissolution rates. The critical pit size r^* is directly proportional to the interfacial tension and inversely proportional to the Gibbs energy of dissolution [174,185]. This results in greater values of r^* (about 10–100 nm) for sparingly soluble biominerals, which always have much higher interfacial free energy values in aqueous solution than soluble salts (see above). Since sparingly soluble salts often have sizes in this critical range, they may thus be protected from dissolution because the reaction in the (relatively wide) metastable region is significantly inhibited [184]. Similarly, the growth of tiny apatite crystals is rarely observed at low supersaturations due to extremely slow growth rates which can also be attributed to a kinetic size effect [184]. In addition, it has been speculated that this crystallite size effect in the dissolution reactions could provide a route for the synthesis of nanoparticles of sparingly soluble salts, whose sizes could be adjusted by changing the dissolution conditions [184].

An equation similar to Equation (1.11) has been discussed in a recent study of calcite growth [4] since steps (growth) and pits (dissolution) have similar roles and features [187]. Both of them are unstable if they are smaller than the critical size, i.e. unstable steps do not advance and unstable pits disappear from the surface [4,187,192]. The inverse scaling of the critical step length with supersaturation is a prediction of the Gibbs–Thomson effect, according to which steps of high curvature at corners should be at equilibrium with the adjacent supersaturated solution [4].

It is noteworthy that the Ostwald–Freundlich model (i.e. the formulation of the Gibbs–Thomson effect for liquid vapour systems applied to solid–liquid systems),

which states that finer particles have a higher solubility and should therefore dissolve faster, is in apparent disagreement with these constant-composition studies of dissolution kinetics [154,184,186,187]. While some researchers observed an increase in solubility upon introduction of small SrSO_4 particles into solutions saturated with larger crystals [193], others did not find any further dissolution when HAP nanoparticles were added to ‘dissolution terminated’ suspensions of larger crystals [154]. The latter are probably in a ‘dynamically stable’ but thermodynamically metastable state that can be maintained for long periods [184]. However, since thermodynamics requires that dissolution cannot stop in an absolute sense, below this critical undersaturation the rate does not necessarily go to zero but will rather be limited by the much slower nucleation or spiral dissolution mechanism so that equilibrium is eventually attained [185]. Schindler *et al.* have carefully measured the solubility constants of CuO and $\text{Cu}(\text{OH})_2$ and determined the influence of molar surface area upon solubility [194]. For coarse solids, the solubility of the hydroxide was found to be ca. 10 times greater than that of the oxide, so that the latter is the more stable phase. However, if the solids are very finely divided, CuO becomes less stable than $\text{Cu}(\text{OH})_2$ (see also Figure 5.23 in [195]). This free energy crossover as a function of particle size, which results from the larger interfacial energy of the oxide as compared to the hydroxide, is in exact analogy to the results derived from calorimetric studies [16,17] and puts some confidence in the relationship between solubility and particle size. Nevertheless, it has been suggested that the thermodynamic basis of the Ostwald–Freundlich model is questionable [177,196], whereas others have derived it in a rigorous way [193,197] and discuss the significance of the surface energy parameter so obtained [176].

In any event, the studies on the dissolution mechanism of sparingly soluble minerals discussed above have again confirmed the long standing recommendation [6] that large crystallite size and long equilibration times are required to attain true equilibrium solubility conditions [192].

1.4.2 NEW MECHANISMS OF BIOMINERALIZATION

While some proteins inhibit pathological calcification, e.g. matrix GLA protein in blood vessels [198] or osteopontin in urine [199], many other proteins promote normal biomineralization of e.g. bone, cementum and dentin by controlling nucleation, growth kinetics, morphology and orientation of the constituent inorganic crystals [1,200]. A topical review has described a number of proteins associated with biominerals [200].

Various mechanisms of biomineralization have been proposed and discussed extensively. A widely accepted view is that the organic matrix or molecules in solution induce nucleation on certain crystal faces (through lowering the Gibbs energy of nucleation by reducing the interfacial energy [1]) and thus control the crystal structure by geometric (epitaxial) matching and stereochemical recognition [201–203]. It has been suggested that these interactions are dynamic, originating

from subtle differences in the kinetics of these recognition processes on different crystal faces rather than from irreversible binding on one set of symmetry related surfaces [203]. The effect of a new heterogeneous nucleation model on the structural correlation at the interface between biomineral and substrate has been investigated recently and a 'supersaturation driven anti-templating' mechanism has been proposed [204]. This mechanism implies that low supersaturations lead to good structural match and consequently to ordered and compact biomineral structure, whereas high supersaturations result in structural mismatch and therefore in disordered and porous biomineral structure [204]. It has been found that polyelectrolytes like poly-L-aspartate, which are structural and functional analogues of subdomains of biomineralization controlling proteins, act as crystal nucleators when immobilized on Ge surfaces, nucleating (less stable) OCP and (more stable) HAP at high and low supersaturations respectively (indicating kinetic control) [205]. Conversely, these polyelectrolytes inhibit crystal growth when free in solution [205] and have therefore been used as biodegradable inhibitors of Ca salt deposition in industrial processes [206].

Whereas HAP crystallization from simulated blood plasma onto Langmuir monolayers of arachidic acid has been described [207], other researchers have reported homogeneous nucleation of calcium phosphates although self assembled monolayer substrates mimicking bone organic matrices were present [208]. These nuclei started to grow in solution and only deposited as apatite on the surface in a second period of crystal growth, allegedly resembling early stages in bone mineralization in vesicular compartments [208]. Various simulations of interfacial control of mineral nucleation on Langmuir monolayers have been reported [209–211].

Recently, a new mineralization process that proceeds via an amorphous, polymer induced liquid-phase precursor (PILP process) has been proposed [169], in which polyelectrolytes like poly-L-aspartate (see above) sequester ions and induce a liquid–liquid phase separation, forming droplets of ca. 2–4 microns (PILP phase) prior to mineralization of various Ca salts. Similar phenomena involving microscopic liquid–liquid phase separation have been found to induce silica mineralisation [212], while other unusual morphologies involving aqueous polymer solutions have been described [213]. The hypothesis that the fluidic nature of the PILP phase is relevant to normal and pathological biomineralization is discussed in detail in Chapter 4 of this book.

Current crystallization studies have employed (i) *in situ* atomic force microscopy (AFM), which enables the visualization of these processes in real time [170,171,199,214,215]; and (ii) highly sensitive constant composition techniques, which provide reliable rates of crystal growth [20,170,171,199]. These studies have suggested another, different mechanism of biomineralization, which potentially provides insights into the prevention and therapy of pathological crystallizations such as renal stone disease. For instance, it is well known that urinary citrate deficiency is a predisposition of calcium oxalate monohydrate (COM) stone formation. It has been shown by AFM and molecular modelling [171,199] that

the inhibitory action of citrate on COM growth does not occur via adsorption on the crystal face but is rather due to a dynamic process of selective binding to atomic steps (formed along dislocation lines for crystal growth) on one specific crystal face, which results in step pinning, step edge roughening, anisotropic step kinetics and consequent crystal growth inhibition and shape modification. Since citrate leaves other crystal faces to grow uninhibited, additional therapeutic agents such as osteopontin, which affects the steps on different faces [199], may be needed for optimal prevention of kidney stone disease [171]. A similar, ‘step control’ model has been suggested in a study of the formation of chiral morphologies through selective binding of amino acids to calcite surface steps, which changes the step edge free energies and therefore the step dynamics, resulting in altered rates of attachment and detachment of calcite species at the surface of the mineral [214]. In another *in situ* AFM study, the recovery of surfaces from impurity poisoning has been investigated [215]. It has been shown that the resurrection of growth out of the ‘dead zone’ (a regime of low supersaturation where growth ceases) proceeds via the propagation of macrosteps (bunches of monolayer steps), which ‘overrun’ the elementary steps that are blocked by the impurities, on a timescale comparable to that of impurity adsorption [215].

Another, new biomineralization mechanism has been proposed [170] based on AFM observations of the crystallization of brushite (DCPD), which is regarded as a precursor in the biological formation of apatite and is found in developing bone, immature dentine and in kidney stones. Using a sensitive constant composition method [20], brushite growth has been studied in the absence and presence of citrate, which has been recognized as an effective inhibitor. However, unlike to the study on citrate inhibition of COM crystallization described above [171,199], neither the step morphology nor the kinetics is affected by citrate. In contrast, citrate dramatically reduces the step density in an anisotropic manner, leading to a corresponding decrease in the bulk growth rate, while the step velocity and morphology are virtually unchanged. The number of steps being formed at surface dislocations depends on the supersaturation and the free energy of the step edge which can be correlated with the interfacial tension between brushite surfaces and the solution. The latter has been measured [170] and found to increase with citrate concentration. The corresponding increased free energies of the step edge imply that the critical length for spontaneous step growth, related to the terrace width for the steps, increases and the formation of new steps becomes retarded. Macroscopically, this is manifested by longer induction times for homogeneous nucleation measured in the presence of citrate, which indicates that this carboxylate-rich compound is a nucleation inhibitor. The authors [170] emphasise that their results are in contrast to the model of epitaxial control of biomineralization as proposed by Mann [1,202], which suggests that energy barriers are reduced by Ca–carboxylate interactions and would thus imply a promotion of the nucleation in the present case.

Tang *et al.* [170] have suggested that approaches involving surface adsorption, e.g. by employing a Langmuir model which had been successfully applied in

earlier growth kinetics and adsorption studies [179,205,216], may be misleading in these cases. Since such approaches have also been used in the industrial context [217], the new findings outlined here will not only be important for improving our understanding of the mechanisms of biomineralization but may also help to optimise large-scale industrial crystallization processes.

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2 Mechanisms of Renal and Salivary Calculi Formation and Development

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2.1 INTRODUCTION

A general classification of most common renal calculi (calcium oxalate, phosphate and uric acid stones) based on their formation mechanism is presented. The main etiological factors that enable their development are discussed considering present knowledge on calcium oxalate, insoluble urinary phosphates and uric acid crystallization and the fine structure of respective renal stones. The general formation mechanism of salivary calculi (sialolithiasis) is also discussed.

2.1.1 RENAL LITHIASIS

Renal lithiasis (renal calculi) affects a wide sector of population, between 4 and 15 % approximately, and it has been classified as one of the illnesses that can cause much pain to human beings. Between 10 and 15 % of the renal calculi require surgical treatment and between 20 and 30 % hospitalization [1–4]. This situation implies a high economic cost that is calculated, for example, in thousands of millions of dollars per year in the USA.

Considering these circumstances, it is surprising to be in the 21st century with still such a high and even increasing incidence of this illness in developed countries. It should be admitted, however that important advances have taken place in the surgical elimination of calculi, this is, percutaneous nephrolithotomy and extracorporeal shock waves lithotripsy, both of them being less aggressive than the classic surgery [5,6]. Nevertheless, it is a very well known fact that between 50 and 70 % of lithiasic individuals will develop a new calculus in a period of less than five years if they do not undergo appropriate prophylactic treatments [7]. Therefore, it is evident that the problem created by renal calculi does not reside exclusively in its elimination, because, independently from the procedure used for this purpose, the alterations

responsible for the formation of the calculus remain without being solved and therefore the risk of the genesis of new calculi persists [8]. In this sense, knowledge of the causes that originate the lithiasic episode is essential for the correct diagnosis and treatment of the illness. However, it should be noted that the protocols of diagnosis and treatment are not standardized so that there is still some confusion and unclear scientific information, which, without doubt, explain the high incidence and prevalence of renal lithiasis.

The explanation of this unsatisfactory state which is surprising if progress in the knowledge of other diseases of much more recent discovery is considered, resides in the very nature of renal lithiasis that implies the formation of solid structures in which crystals and organic matter coexist, and that are generated in a complex liquid medium such as urine, inside renal cavities with walls constituted by cellular structures [9,10]. Thus, there are three fundamental reasons which explain this late and insufficient development of knowledge of the illness.

First of all, the understanding of the detailed mechanisms of the genesis of a renal calculus requires knowledge about renal physiology and biochemistry (in the classic sense of the term), but it is also necessary to collect much knowledge about crystallization, which is not usually common in the environment of health sciences. Thus, generally in medical studies, only clinical treatment of lithiasis is superficially explained, without going into details of the pathophysiology. In urology, specialty surgical aspects are more deeply explained but the study of molecular mechanisms is not usually approached.

Secondly, renal lithiasis is a chronic pathology, of multifactorial origin, in which environmental (climatic, dietary, behaviour...) and genetic factors are mixed. Such multifactorial origin can allow one, by means of an appropriate evaluation, to find a solution that avoids the use of complex drugs which, besides the problems associated to their potential secondary effects, present the problems of the chronicity of the treatment, that is, a treatment practically for life. From this aspect a scarce pharmacological need is derived, generating little interest in the big pharmaceutical companies that do not foresee any important business, and due to the lacking economic interest for them, do not motivate investigation in this field.

Thirdly, although genetics (and molecular biology in its classic sense) participate among the factors implied in this pathology, its weight is not at all decisive [11]. Only one lithiasis type exists, in fact not very common (cystine lithiasis that only amounts to 1 % of all cases) which is bound to a clearly identified genetic alteration, although many carriers do not manifest the illness, as with many other illnesses of genetic origin [12]. Nevertheless, these circumstances obviously separate renal lithiasis from the research trend that is at present fashionable and that has many addicts.

Renal lithiasis can be defined as an alteration in the balanced conditions of crystallization of the urine. The factors that participate in the formation of crystals can be diverse and for this reason renal lithiasis is clearly a multifactorial pathology. The time required to generate a crystal depends fundamentally on its nature, on the supersaturation of the solution (excess salts in the solution; driving force of

the crystallization; thermodynamic factor), on the presence of pre-existing solid particles (the so-called heterogeneous nucleants; kinetic factor) and on the level of inhibitors of the crystallization (kinetic factor) [13]. These last are substances that due to their chemical structure interact with the nucleus or the faces of the crystal, interfering notably in their formation and/or development and slowing down or preventing the crystallization processes. As a consequence, crystallization processes depend on the balance between thermodynamic and kinetic factors. All human urine is supersaturated with respect to calcium oxalate [14], in such a way that the degree of supersaturation is higher for hypercalciuric and/or hyperoxaluric individuals. Depending on the value of urinary pH, the urine can also be supersaturated with respect to other substances such as calcium phosphates (hydroxyapatite, brushite, for urinary pH higher than 6) or uric acid (for urinary pH lower than 5.5). The human urine can also contain a wide variety of heterogeneous nucleants as protein aggregates, cellular debris, bacteria, etc.; in this aspect the nucleant capacity of the altered renal epithelia should be also considered. These circumstances mean that in any urine different substances can crystallize depending on the time that elapses before their emission (micturition). Fortunately, most urines do not form crystals during their time of residence in the high urinary tract due to an appropriate balance between thermodynamic and kinetic factors. It is evident that time is a very important variable in the crystal formation processes, since kinetic factors (heterogeneous nucleants and inhibitors of the crystallization) play a decisive role [13]. Thus, it is clear that when increasing the residence time of the urine in the urinary tract (mainly in the upper tract), the possibility of developing crystallization processes that lead to the formation of renal calculi is increased and therefore the existence of renal cavities of low urodynamic efficacy constitutes an important risk factor for urolith development. When the development of crystals takes place in the urinary bladder, these are usually eliminated without difficulty as asymptomatic crystalluria.

2.1.2 SIALOLITHIASIS

Sialolithiasis is a common disease of salivary glands characterized by the obstruction of salivary secretion by a calculus. This is associated with pain and inflammation and in some cases to an infection of the affected gland. This disease corresponds to 30 % of the salivary pathologies and is more frequent in adults (0.1–1.0 % of the population) than in children [15–18].

These calculi generally consist of mixtures of different calcium phosphates (mainly hydroxyapatite and carbonate-apatite) together with an organic matrix [16,19–22]. When occasionally infection was present, ammonium and magnesium can be also found.

The etiology of these calculi is little known and their exact mechanism of formation is unknown [17].

2.2 CLASSIFICATION OF RENAL CALCULI AND FORMATION MECHANISMS

Considering the nature of the major components of renal calculi, the presence of minor substances and their location, as well as the etiological factors that can be deduced from the macro- and the micro-structure of the renal calculi, we have classified them into 11 groups that are indicated in Table 2.1 [23]. The main urinary alterations that are frequently associated to each renal calculus type are shown in Table 2.2 [23]. The metabolic risk values of urinary parameters for renal lithiasis are indicated in Table 2.3 [23].

Table 2.1 Renal stone classification. (Reprinted from *Clin. Chim. Acta*, Vol. 322, F. Grases, A. Costa-Bauzá, M. Ramis, V. Montesinos and A. Conte, Simple classification of renal calculi closely related to their micromorphology and etiology, 29–36, 2002, with permission from Elsevier.).

Type and main component	%	Other important structural characteristics
Calcium oxalate monohydrate papillary calculi	12.9	– core constituted by COM/OM (60.7 %) – core constituted by HAP/OM (39.2 %) – size^a: 2–7 mm
Calcium oxalate monohydrate unattached calculi (formed in renal cavities)	16.4	– core constituted by OM (63.3 %) – core constituted by HAP (29.9 %) – core constituted by uric acid (6.7 %) – size^a: 2–15 mm
Calcium oxalate dihydrate unattached calculi	33.8	– containing little amounts of HAP among COD crystals (55.2 %) – only COD and little amounts of OM (44.8 %) – can contain variable amounts of COM, even 100 %, but it comes from the transformation of COD – size^a: 2–15 mm
Calcium oxalate dihydrate/hydroxyapatite mixed unattached calculi	11.2	– alternative COD/HAP layers (39.6 %) – disordered COD/HAP deposits (60.4 %) – size^a: 3–15 mm
Hydroxyapatite unattached calculi	7.1	– containing minute amounts of COD (54.9 %)

		– containing only HAP and OM (45.1 %) – size^a: 2–15 mm
Struvite infectious calculi	4.1	– also contain large amounts of HAP and OM – size^a: 5–50 mm
Brushite unattached calculi	0.6	– frequently also contain little amounts of HAP – size^a: 3–15 mm
Uric acid unattached calculi	8.2	– mainly anhydrous uric acid (40.7 %) – mainly dihydrate uric acid (49.0 %) – uric acid / urates mixed calculi (8.8 %) – size^a: 1–20 mm
Calcium oxalate /uric acid mixed calculi	2.6	– papillary (12.7 %) – unattached (non papillary) (87.3 %) – size^a: 3–20 mm
Cystine unattached calculi	1.1	– also contain minute amounts of OM – size^a: 3–30 mm
Unfrequent calculi	1.9	– OM as main component (32.6 %) – medicamentous (6.1 %) – post SWEL residues (10.2 %) – calcium carbonate (14.3 %) – artefacts (36.7 %)

^a values of size approximately correspond to the range of the larger dimension of calculi.

COM: calcium oxalate monohydrate
COD: calcium oxalate dihydrate
OM: organic matter
HAP: hydroxyapatite
SWEL: shock waves extracorporeal lithotripsy

Table 2.2 Urinary etiologic factors and their relation to the calculus type (comparison with a healthy people control group). (Reprinted from *Clin. Chim. Acta*, Vol. 322, F. Grases, A. Costa-Bauzá, M. Ramis, V. Montesinos, and A. Conte, Simple classification of renal calculi closely related to their micromorphology and etiology, 29–36, 2002, with permission from Elsevier.).

Type of calculi	Main urinary alterations more frequently found
Calcium oxalate monohydrate papillary	– deficit of urinary crystallization inhibitors (citrate (36 %)) – urinary pH > 6.0 (when HAP was present (50 %)) (the development of these calculi must imply also some damage of the papillary urothelium)
Calcium oxalate monohydrate unattached (formed in renal cavities)	– deficit of urinary crystallization inhibitors (citrate (47 %)) – urinary pH > 6.0 when HAP was present (58 %) or pH < 5.5 when uric acid was present (82 %) (obviously the existence of cavities with low urodynamic efficacy favours the formation of these calculi)

Table 2.2 (Continued).

Type of calculi	Main urinary alterations more frequently found
Calcium oxalate dihydrate	– hypercalciuria (60 %) – deficit of urinary crystallization inhibitors (citrate (50 %)) – urinary pH > 6.0 (when HAP was present (62 %)) (the existence of cavities with low urodynamic efficacy favours the formation of these calculi)
Calcium oxalate dihydrate/ hydroxyapatite mixed calculi	– hypercalciuria (69 %) – urinary pH > 6.0 (68 %) – hypocitraturia (54 %) (the existence of cavities with low urodynamic efficacy favours the formation of these calculi)
Hydroxyapatite	– urinary pH > 6.0 (75 %) – hypocitraturia (70 %) (the existence of cavities with low urodynamic efficacy favours the formation of these calculi)
Struvite infectious	– urinary infection
Brushite	– urinary pH > 6.0 – deficit of urinary crystallization inhibitors (the existence of cavities with low urodynamic efficacy favours the formation of these calculi)
Uric acid	– urinary pH < 5.5 (80 %) – hyperuricuria (41 %) (the existence of cavities with low urodynamic efficacy favours the formation of these calculi)
Calcium oxalate/uric acid mixed calculi	– deficit of urinary crystallization inhibitors (citrate (54 %)) – urinary pH < 5.5 (46 %) – hyperuricuria (54 %)
Cystine	– hypercystinuria – urinary pH < 5.5 (the existence of cavities with low urodynamic efficacy favours the formation of these calculi)

HAP: hydroxyapatite

Independent of their chemical composition, renal calculi can be classified as a whole in two big categories: calculi formed on the renal walls (attached to the renal papillae) in which the point of attachment to the epithelium is clearly distinguished, and calculi developed in renal cavities (without area of attachment to the epithelium) (see Figure 2.1) [24]. A detailed description of the mechanism of formation of the most common renal calculi will be put forward as follows.

Table 2.3 Metabolic risk values for urolithiasis. (Reprinted from *Clin. Chim. Acta*, **322**, F. Grases, A. Costa-Bauzá, M. Ramis, V. Montesinos, and A. Conte, Simple classification of renal calculi closely related to their micromorphology and etiology, 29–36, 2002, with permission from Elsevier.).

Serum	Risk	Urine	Risk
Ca (mg/dl)	> 10.2	Ca (mg/24 h)	> 250 (women) > 300 (men)
P (mg/dl)	> 4.5	Mg (mg/24 h)	< 70
Mg (mg/dl)	< 1.8	P (mg/24 h)	> 1200
Uric acid (mg/dl)	> 6.5	Uric acid (mg/24 h)	> 600 (women) > 800 (men)
Creatinine (mg/dl)	> 1.2	Creatinine (mg/24 h)	> 2000
		Oxalate (mg/24 h)	> 40
		Citrate (mg/24 h)	< 350
		Phytate (mg/24 h)	< 1.0
		Cystine (mg/24 h)	> 20
		pH	< 5.5 > 6.0

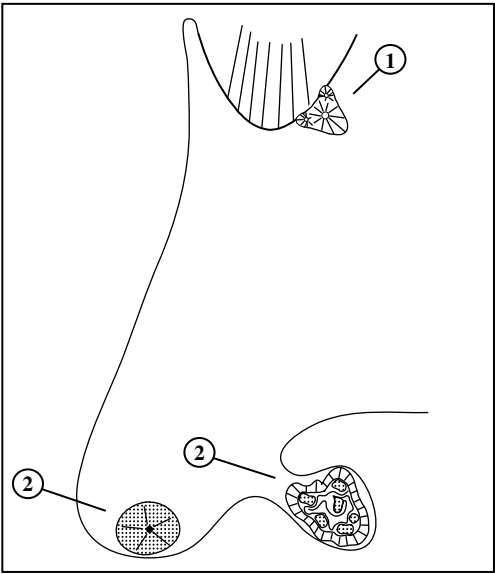


Figure 2.1 Scheme of renal calyx and location of (1) papillary calculi and (2) unattached calculi in lower calyx or in a low urodynamic efficacy cavity.

2.2.1 CALCIUM OXALATE RENAL CALCULI

As for all renal calculi, their formation take place as a consequence of the unfortunate combination of several factors. It is therefore convenient, in order to discuss its etiology, to distinguish two large groups, considering the majority component:

- (I) Calcium oxalate monohydrate renal calculi
- (II) Calcium oxalate dihydrate renal calculi

I Calcium oxalate monohydrate renal calculi

The calcium oxalate monohydrate renal calculi usually generate in individuals with normal urinary excretions of calcium. To properly discuss their etiology it is necessary to classify them in turn, in two subgroups:

- (I.1) Papillary calcium oxalate monohydrate renal calculi: they are usually semispherical, about 2 or 3 mm diameter, with one convex surface sometimes lobular, the other one remaining attached to the renal papillae concave (Figure 2.2) [25].
- (I.2) Non-papillary calcium oxalate monohydrate renal calculi: spherical, usually presenting several lobules (muriform aspect, like a blackberry) with a diameter larger than 1 cm, without presenting areas of union with the urothelium (Figure 2.3) [26].

The papillary calcium oxalate monohydrate renal calculi begin their formation on the renal papillae, in areas where the glycosaminoglycans antiadherent layer that covers and protects it is reduced or destroyed as a consequence of some cellular dysfunction or external attack [27]. If the damage and/or cellular destruction is considerable (for example, necrosis caused by analgesics) [28], the organic detritus can act as an inductor (heterogeneous nucleant) of the calcium oxalate crystals that will begin to grow and will constitute the future core of the calculus (Figure 2.4). It is important to consider that, although any urine is supersaturated with regard to calcium and oxalate, the normal concentrations of these substances are not high enough to induce the formation of calcium oxalate crystals (homogeneous nucleation), so that they need a nucleus that will present a different composition than the rest of the calculus [25]. When the destruction or cellular damage is not enough to induce the formation of calcium oxalate crystals, other substances that are deposited under appropriate conditions on the altered areas (low protected) of the renal papillae can exist, acting later as inducers of the calcium oxalate crystal growth. Among these substances the calcium phosphates (brushite and hydroxyapatite) should be mentioned for values of urinary pH higher than 6.0 or due to subepithelial calcifications that by disruption of epithelium will contact with urine (Randall's plaque). In all the outlined situations, it is very important to consider the action of the inhibitors of the crystallization. Thus, they can impede the appearance of the inducers of calcium oxalate crystallization, or even the calcium oxalate growth

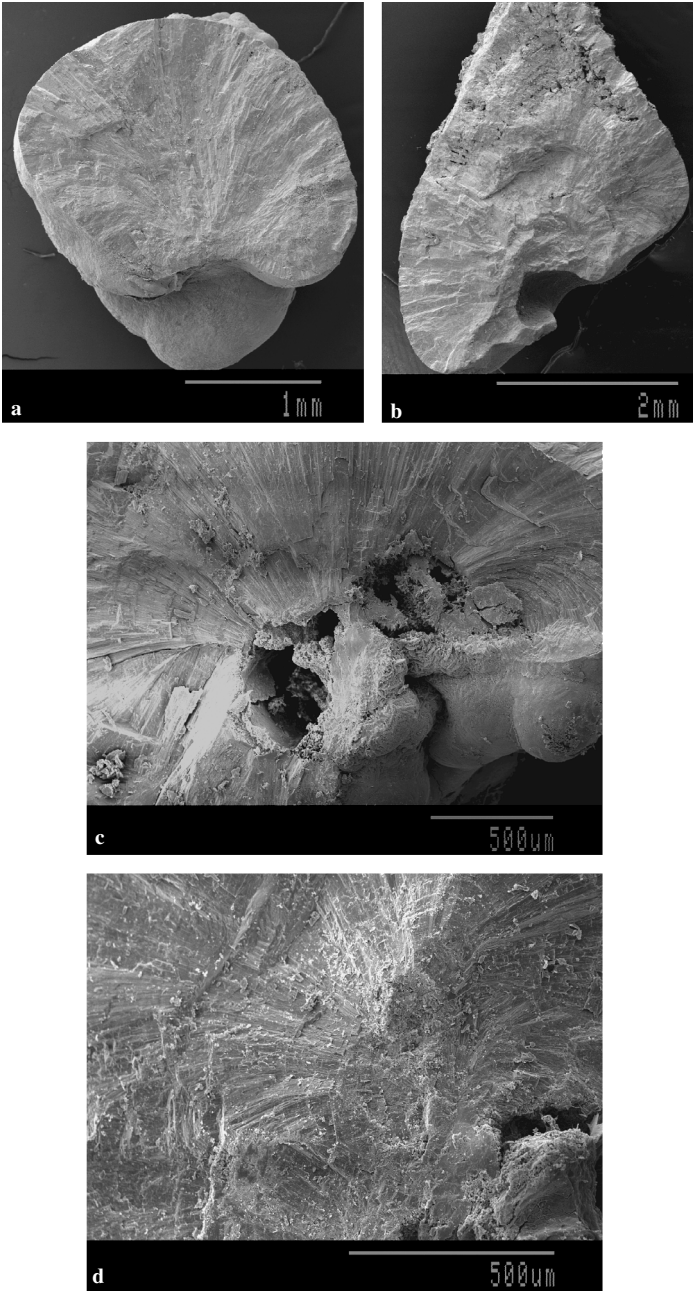


Figure 2.2 Scanning electron microscopy images of: (a, b) typical calcium oxalate monohydrate papillary calculi sections; (c, d) detail of the core and point of attachment to the renal papillae of calcium oxalate monohydrate papillary calculi.

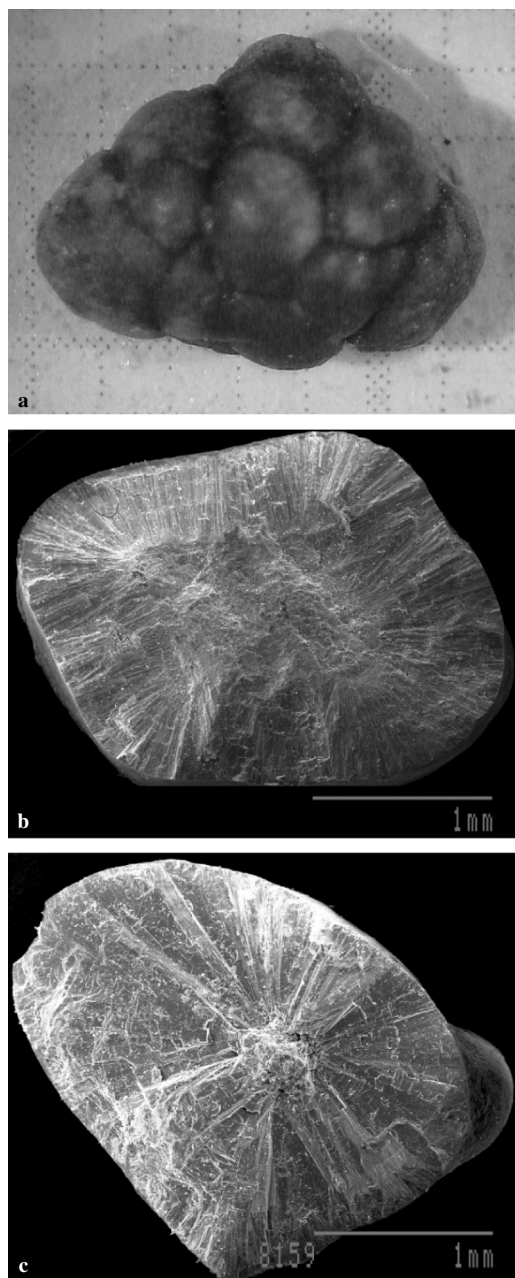


Figure 2.3 (a) Stereoscopic microscopy image of a calcium oxalate monohydrate non-papillary calculus; (b, c) scanning electron microscopy images of typical calcium oxalate monohydrate non-papillary calculi sections (no point of attachment to the papillae can be detected).

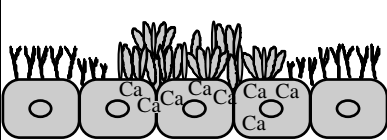
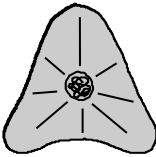
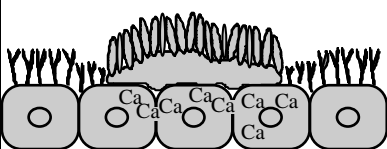
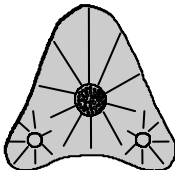
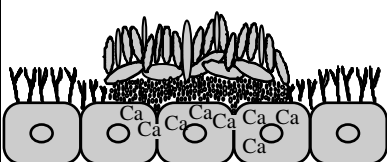
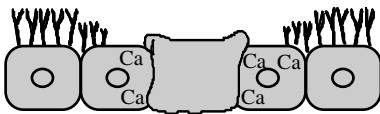
Core type and etiology	Inner structure	Mechanism of first step formation
Core = calcium oxalate monohydrate primary aggregates - slight severe injury - deficit of inhibitors		
Core = organic matter as heterogeneous nucleant of calcium oxalate monohydrate crystals - slight severe injury - proteins in urine - deficit of inhibitors		
Core = hydroxyapatite - slight severe injury - pH > 6.0 - deficit of inhibitors		
Core = organic matter and hydroxyapatite - severe injury (Randall's plaque) - pH > 6.0 - deficit of inhibitors		
Core = organic matter - severe injury - deficit of inhibitors		

Figure 2.4 Diagram of the characteristics, etiology and mechanisms of first step formation of the different calcium oxalate monohydrate papillary calculi.

on these inducers. It is clear, therefore that a deficit in the excretion of inhibitors of crystallization constitutes another important risk factor for the development of this type of calculi. Among the inhibitors that human beings excrete naturally, it is necessary to highlight citrate and phytate [29–31].

Once a small disordered aggregate of calcium oxalate crystals of a size of the order of 0.5 mm has been constituted (heart of the calculus) attached to the papillary wall, it is practically impossible to stop the process, since on this mass calcium oxalate monohydrate crystals begin to grow in a perpendicular direction to the surface (columnar area of the calculus, Figure 2.4) and sooner or later they will constitute the whole calculus.

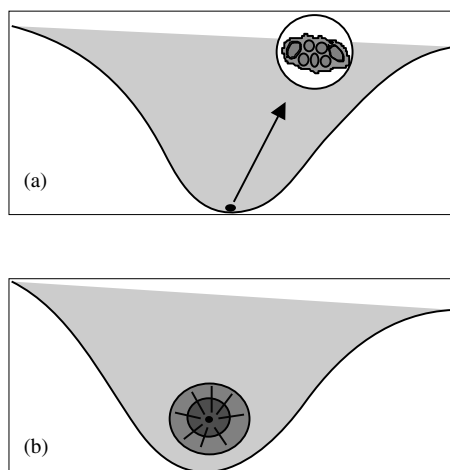


Figure 2.5 Diagram of the mechanism of formation of calcium oxalate monohydrate non-papillary calculi. (a) retention of a heterogeneous nucleant (organic matter, hydroxyapatite, uric acid, etc.) in a low urodynamic efficacy cavity and core formation; (b) columnar calcium oxalate monohydrate crystal development on the initially formed core.

The non-papillary calcium oxalate monohydrate renal calculi present, in general, an internal crystalline structure clearly different to the papillary ones. They present a central area constituted by the heterogeneous nucleant responsible for their formation (organic matter, hydroxyapatite, uric acid, drug crystals, etc.) [23]. On this central core, columnar calcium oxalate monohydrate, which will constitute the corresponding calculus, grows (Figure 2.5). Since urinary concentrations of calcium and oxalate are not enough to justify the formation of calcium oxalate crystals, the existence of promoters (heterogeneous nucleants) of its formation is indispensable. As for papillary calcium oxalate monohydrate renal calculi, among these substances it is necessary to mention the organic detritus, calcium phosphates (for values of urinary pH higher than 6.0) and uric acid (for urinary pH lower than 5.5). In addition the role that crystallization inhibitors can play is very important, hence their deficit constitutes a risk factor for this type of renal calculi development [32].

II Calcium oxalate dihydrate renal calculi

The calcium oxalate dihydrate renal calculi usually generate in individuals with abnormally high urinary excretion of calcium and/or oxalate (hypercalciuria and/or hyperoxaluria) [33]. The calcium oxalate dihydrate renal calculi appear confined in cavities of low urodynamic efficacy. In this case, the presence of calcium phosphates that will act as active heterogeneous nucleants, and the high concentration of calcium, promote the development of the thermodynamically unstable calcium oxalate dihydrate crystals [34]. The bipyramidal morphology of calcium oxalate

dihydrate crystals impedes its growth in parallel for orderly structure formation, which is the reason these calculi present disordered structures in which the calcium oxalate dihydrate crystals are superimposed and small deposits of calcium phosphates can be detected among them (Figure 2.6). As for calcium oxalate monohydrate, calcium oxalate dihydrate crystals, once formed, can induce the growth of other crystals of the same nature on their faces and edges, favouring the formation of crystalline aggregates (a phenomenon known as primary aggregation) (Figure 2.7). Organic matter of diverse origin can also act as heterogeneous nucleants of calcium oxalate dihydrate crystals [35]. Obviously crystallization inhibitors can also play an important role in avoiding crystal development.

2.2.2 PHOSPHATE RENAL CALCULI

As for all the renal calculi, their formation depends on the combination of several factors, so that to discuss its etiology, it is convenient to perform a classification of them, in accordance with their composition, into the following groups:

- (I) Infection phosphate renal calculi: calculi that can reach considerable sizes (even occupying the whole renal cavity = staghorn calculi) in whose composition magnesium-ammonium phosphate (struvite) and organic matter as main components, accompanied by variable proportions of hydroxyapatite can be detected (Figure 2.8).
- (II) Non-infective calcium phosphate renal calculi:
 - (II.a) Calculi of small size mainly constituted by hydroxyapatite (Figure 2.9)
 - (II.b) Calculi of small size mainly constituted by brushite (Figure 2.10)

I Infection phosphate renal calculi (magnesium-ammonium phosphate)

The bacterial infection of the urinary tract is usually the most common cause of this lithiasis [36,37]. The ureolithic germs (proteus, klebsiellas, pseudomonas, ureoplasma...) usually cause a remarkable increase of the urinary pH ($\text{pH} > 7$) and of the ammonium urinary concentration that favour the precipitation of the magnesium-ammonium phosphate and of the hydroxyapatite. The crystalline mass formed together with the organic detritus (cellular debris, bacteria, mucoproteins...) by simple sedimentation can form important deposits that, by loss of water, will originate the renal calculi (Figure 2.11).

It should also be kept in mind that the presence of renal calculi of calcium oxalate, uric acid, calcium phosphates, etc., can provoke urothelial injuries that can induce infections hence causing an elevation of the urinary pH thereafter favouring the precipitation of magnesium-ammonium phosphate. This would generate mixed calculi of calcium oxalate–magnesium-ammonium phosphate,

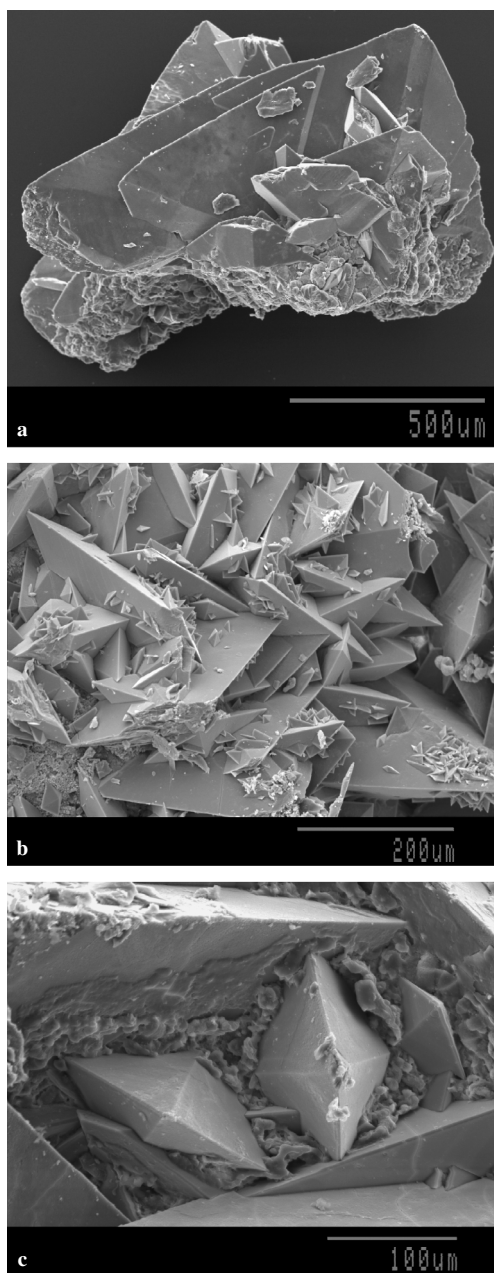


Figure 2.6 Calcium oxalate dihydrate calculi images: (a) general view of a small calculus; (b) detail of calcium oxalate dihydrate crystals. Primary aggregates can be observed; (c) detail of calcium oxalate dihydrate crystals surrounded by hydroxyapatite.

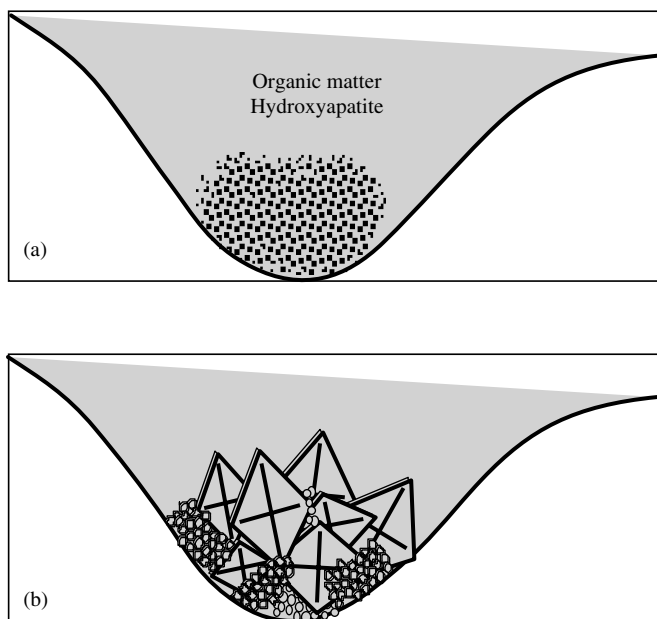


Figure 2.7 Diagram of the mechanism of formation of calcium oxalate dihydrate calculi: (a) retention of organic matter and/or hydroxyapatite in a low urodynamic efficacy cavity; (b) calcium oxalate dihydrate crystals are formed by heterogeneous nucleation and replicate through formation of primary aggregates.

uric acid–magnesium-ammonium phosphate, etc. On the other hand, magnesium-ammonium phosphate infective lithiasis is also related, in more than 50 % of the cases, to hypercalciuria.

II Non-infective phosphate renal calculi

II.a Non-infective phosphate renal calculi of hydroxyapatite

The formation of this type of renal calculus requires the existence of renal cavities with low urodynamic efficacy, combined with urinary pH values higher than 6 and hypomagnesiuria [38,39]. Under these conditions hydroxyapatite microcrystals are generated in the urine that will settle around the walls of the cavity. The successive entrance of material together with compacting by loss of water ends up generating renal calculi whose external morphology will depend on the cavity shape in which they have been formed (Figure 2.12).

II.b Non-infective phosphate renal calculi of brushite

As in the previous case, the formation of this type of renal calculus requires the existence of renal cavities of reduced urodynamics. In this case, however, magnesium urinary concentration is usually normal, the urinary pH oscillates between 6.0 and 7.0 and it is common to detect an inhibitory deficit [38–40].

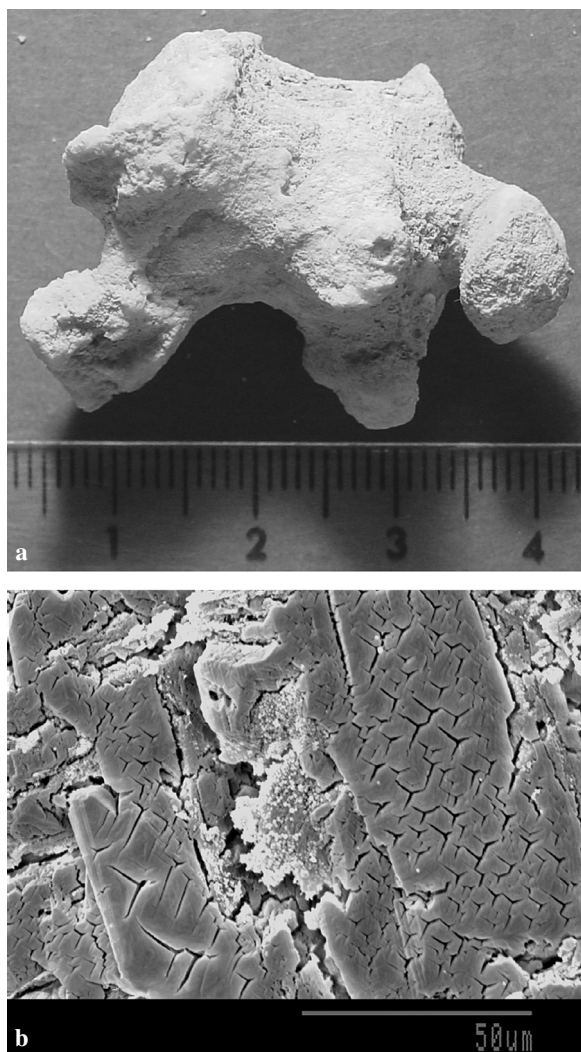


Figure 2.8 Struvite infectious calculi images: (a) general view obtained by stereoscopic microscopy; (b) detail of the surface of a magnesium-ammonium phosphate crystal (struvite), where the typical Y footmark can be observed.

Under these conditions, brushite crystals and hydroxyapatite are generated in urine. Due to the supersaturation values reached, growth of brushite crystals is slow, thus reaching considerable sizes, in such a way that the morphology of these crystals allows them to grow in parallel forming columnar structures (Figure 2.13). Therefore, in this type of calculi, sedimentation and crystalline growth phenomena are combined.

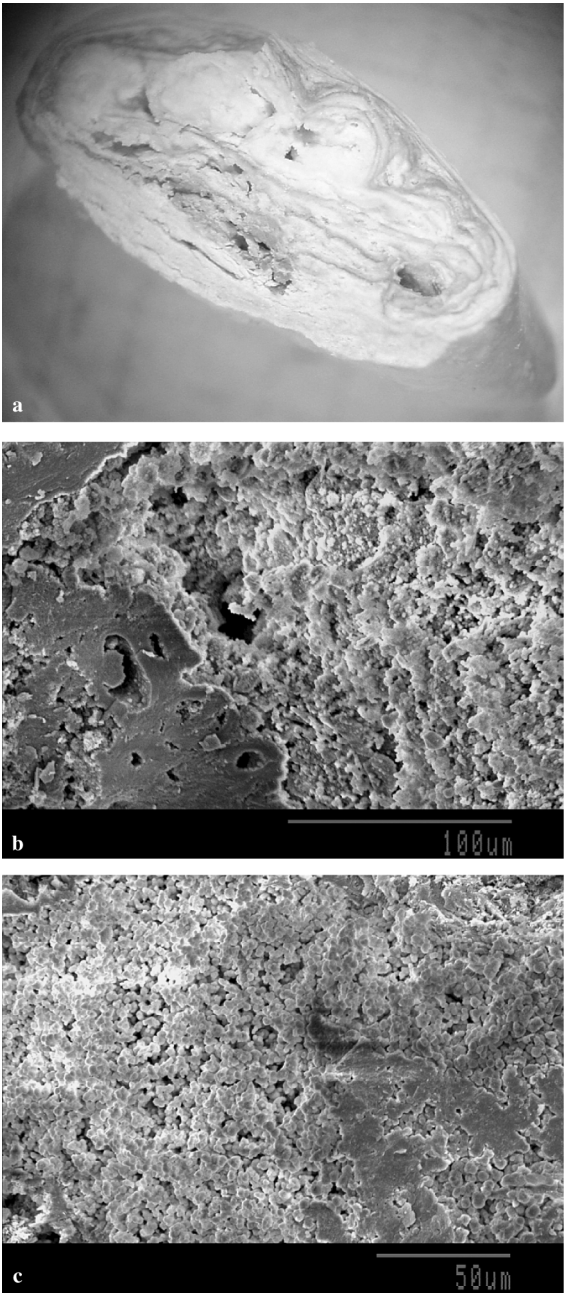


Figure 2.9 Hydroxyapatite renal calculi images: (a) general view obtained by stereoscopic microscopy; (b,c) detail of hydroxyapatite renal calculus obtained by scanning electron microscopy. Spherulites of hydroxyapatite and aspidinic zones can be observed.

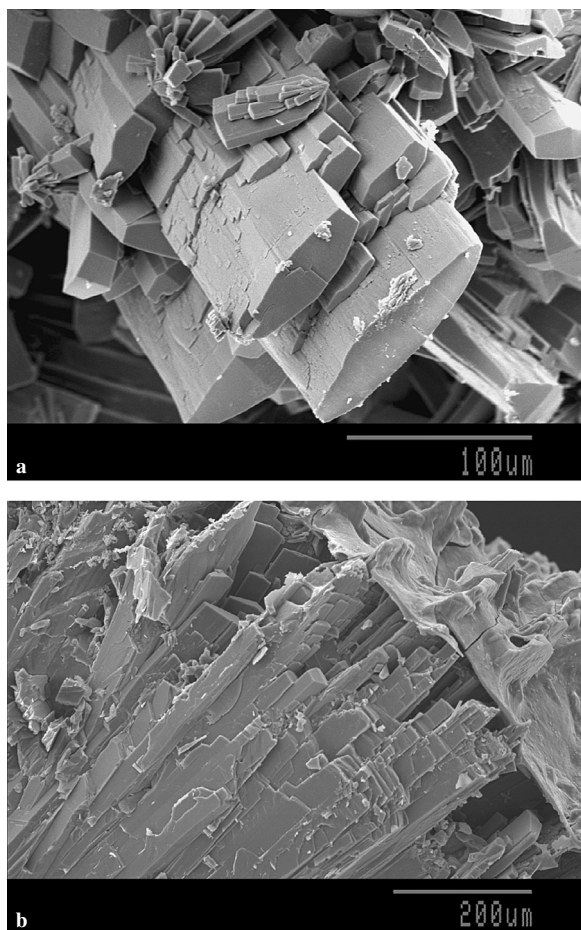


Figure 2.10 Brushite renal calculi scanning electron microscopy images: (a) typical tabular brushite crystals; (b) columnar growth of brushite crystals forming compact and hard structures.

2.2.3 CALCIUM OXALATE DIHYDRATE/HYDROXYAPATITE MIXED CALCULI

This type of calculus is formed when the following factors coincide: existence of renal cavities with low urodynamic efficacy, urine with hypercalciuria (in many occasions due to primary hyperparathyroidism); deficit of crystallization inhibitors, and urinary pH higher than 6.0 [41]. High values of urinary pH in many occasions are due to renal tubular acidosis. The internal structure of these calculi can be completely disordered or present alternate layers of calcium oxalate dihydrate and hydroxyapatite (Figures 2.14, 2.15).

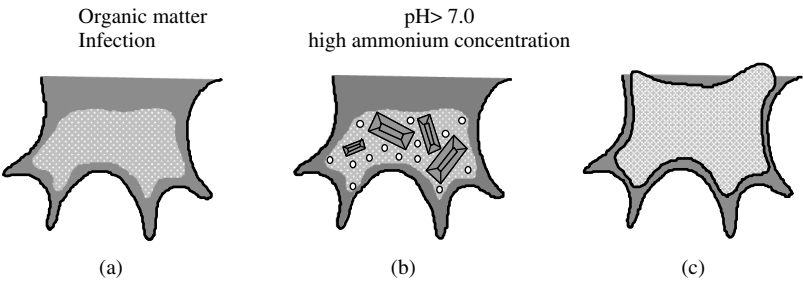


Figure 2.11 Diagram of the mechanism of formation of struvite infectious renal calculi: (a) organic matter (cellular detritus and bacterial debris) forming an initial gelatinous mass that induces massive phosphate salt precipitation; (b) formation of struvite and hydroxyapatite crystals; (c) compacting process and formation of the final solid mass.

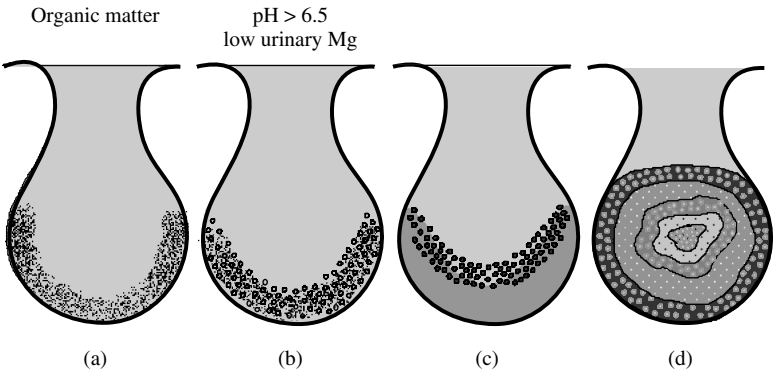


Figure 2.12 Diagram of the mechanism of formation of hydroxyapatite renal calculi: (a) organic matter forming a gelatinous initial mass; (b) hydroxyapatite formation at urinary pH > 6.0 and low urinary magnesium concentration; (c) development of different layers of organic matter and hydroxyapatite; (d) formation of the calculus is stopped when the renal cavity is totally occupied.

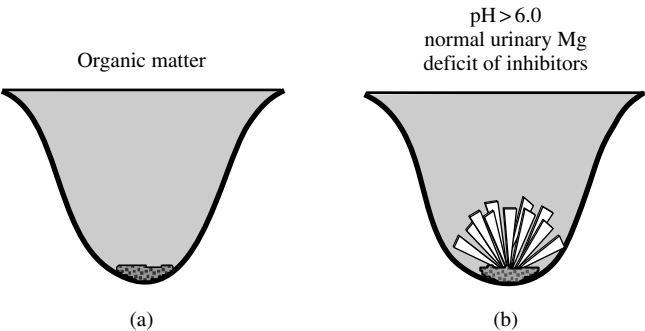


Figure 2.13 Diagram of the mechanism of formation of brushite renal calculi: (a) organic matter is deposited in a cavity; (b) tabular brushite crystals develop on the organic matter in appropriate conditions.

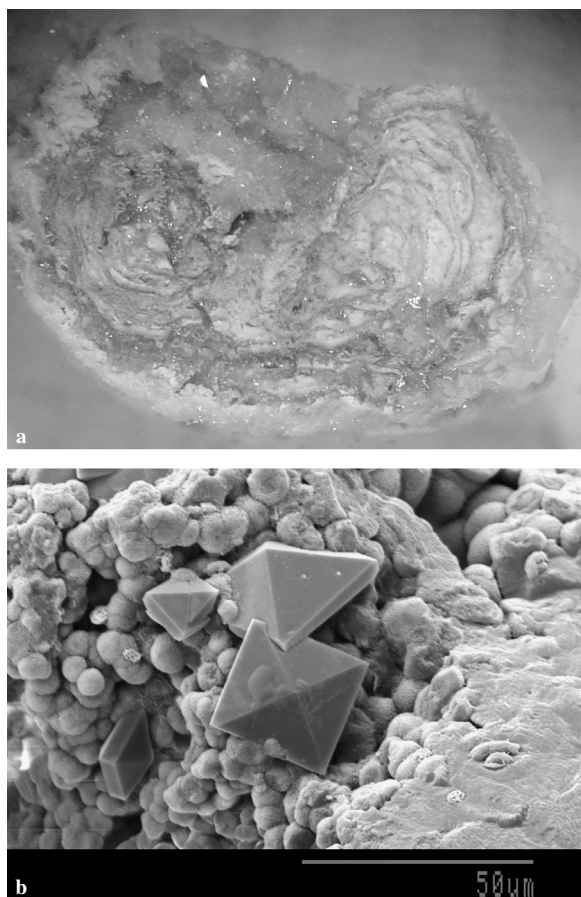


Figure 2.14 Calcium oxalate dihydrate/hydroxyapatite mixed renal calculi images: (a) stereoscopic microscopy view of a section. The multilayer structure can be appreciated; (b) scanning electron microscopy detail where calcium oxalate dihydrate crystals and hydroxyapatite spherulites can be observed.

2.2.4 URIC ACID RENAL CALCULI

The excretion of significant quantities of uric acid in urine, and/or the persistent existence of low urinary pH values ($\text{pH} < 5.5$) cause a high supersaturation of uric acid in urine, that drives to their separation in the form of a solid phase, thus allowing the corresponding renal calculi to generate [42–44]. Nevertheless, there are individuals who excrete large amounts of uric acid in the urine together with low urinary pH values and do not form uric acid renal calculi. This fact is explained by considering that a single factor is rarely able to unchain a lithiasic

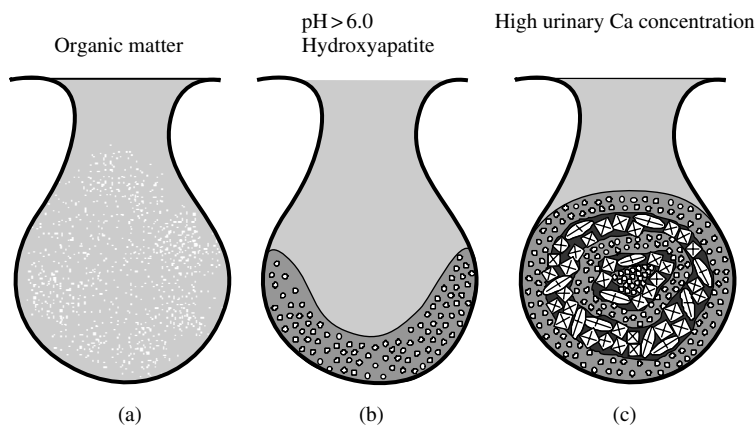


Figure 2.15 Diagram of the mechanism of formation of calcium oxalate dihydrate/hydroxyapatite mixed renal calculi. (a) organic matter forming a gelatinous initial mass; (b) hydroxyapatite formation at urinary pH > 6.0; (c) calcium oxalate dihydrate crystal development is induced by hydroxyapatite and several alternate layers develop until the calculus fills the renal cavity.

episode. Probably only in the case of extreme alterations, is a single factor enough to impel the formation of a renal calculus. In the case of the uric calculogenesis, it is clear that the decisive factor for the formation of the calculus is the excretion of significant quantities of uric acid in urine with low pH, likewise if these factors can be corrected, the problem will be probably eliminated; however, it is also clear that if there were not other adverse circumstances, the problem would surely not be generated. Among the other causes that favour the formation of uric acid renal calculi it is necessary to mention the existence of renal cavities with reduced urodynamic efficacy that would permit the sedimentation, retention and growth of uric acid crystals, thus facilitating the genesis of these renal calculi (Figures 2.16, 2.17).

In the case of the formation of uric acid renal calculi it is also necessary to consider the possible participation of the inhibitors of the crystallization, like glycosaminoglycans and glycoproteins, which although in many cases are not able to impede the crystallization of uric acid due to high supersaturation, at least they can retard or slow down the process [45,46].

Uric acid is not present in renal calculi in a single chemical form. It mainly appears as anhydrous uric acid (thermodynamically stable phase), forming on occasions well-developed crystals (Figure 2.16), this demonstrating that they have been formed through slow growth (low supersaturation) or by recrystallization. Uric acid dihydrate that is formed at higher supersaturations has also been detected. Uric acid dihydrate is, however, a very unstable phase, with relatively fast dehydration, being transformed into the anhydrous form, and generating as a consequence

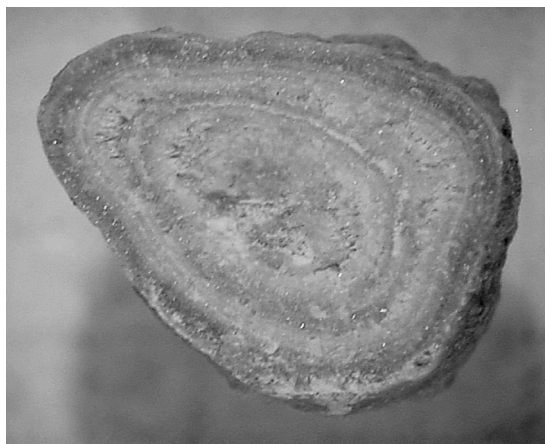


Figure 2.16 Anhydrous uric acid calculus section image obtained by stereoscopic microscopy. A concentric non-columnar structure can be observed.

fragile structures, with cracks and fissures (Figure 2.17) [42,47]. In many renal calculi areas with crystals of anhydrous uric acid and areas with crystals of uric acid dihydrate can be observed. It is also frequent to detect crystals of calcium oxalate, basically monohydrate, among the uric acid crystals, this confirming the capacity that uric acid crystals present to act as a heterogeneous nucleant of calcium oxalate.

When an urinary infection takes place in urine with a high content of uric acid and the urinary pH is lower than 7, ammonium urate can reach high supersaturation values and precipitate before magnesium-ammonium phosphate, thus generating renal calculi in which ammonium urate is the major component (Figure 2.18) [48].

2.2.5 CYSTINE RENAL CALCULI

Cystine is an amino acid that is very insoluble in water at low pH values. For this reason when an abnormally high urinary elimination of cystine takes place and the urinary pH is low, this substance reaches high supersaturation values, precipitates and can eventually generate a renal calculus (Figure 2.19) [49,50]. This alteration has a clearly genetic base and the presence or absence of renal calculi in these cases depends on whether the transmission of the gene is homozygous or heterozygous. This type of renal calculus is not papillary and its formation is basically produced by the combination of sedimentation processes and crystalline growth (a similar mechanism to that of the formation of brushite calculi). In this case it is interesting to highlight that only a certain percentage of individuals with the mentioned genetic alteration and excess cystine

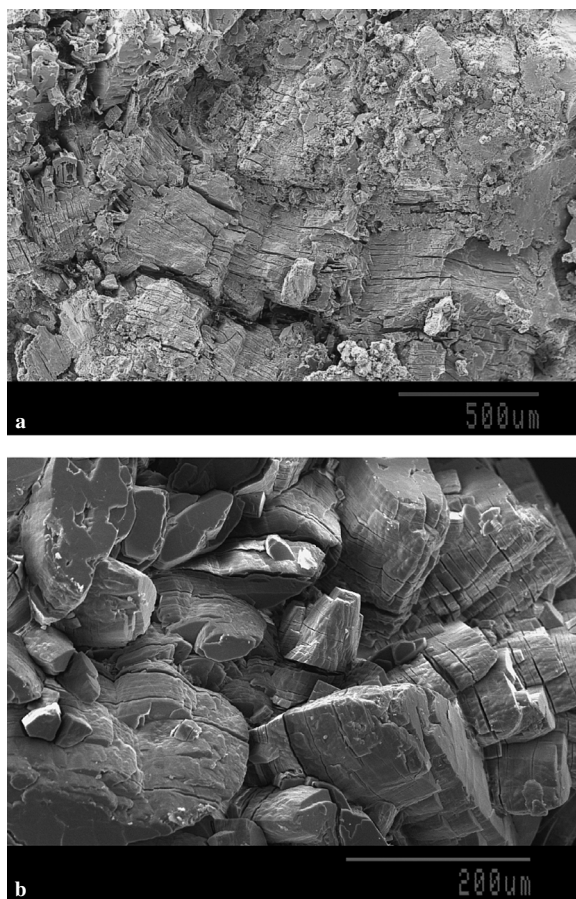


Figure 2.17 Scanning electron microscopy images of dihydrate uric acid renal calculi: (a) general view of a calculus mass; (b) typical cracks due to the water loss can be observed on originally dihydrate uric acid crystals.

in urine (hypercystinuria) actually forms cystine calculi. What can be deduced from this situation? First of all it is very clear that, in spite of the existence of a genetic alteration, directly related to the development of renal calculi, this illness may not appear, so there should be other circumstances involved. On the other hand, when a renal calculus is generated due to a genetic alteration, its formation can be avoided by means of actions that obviously do not require the application of gene therapy. Finally, knowledge of the existence of one of these genetic alterations, prior to the development of the first renal calculus, is of great diagnostic value, since this could completely prevent the development of these stones.

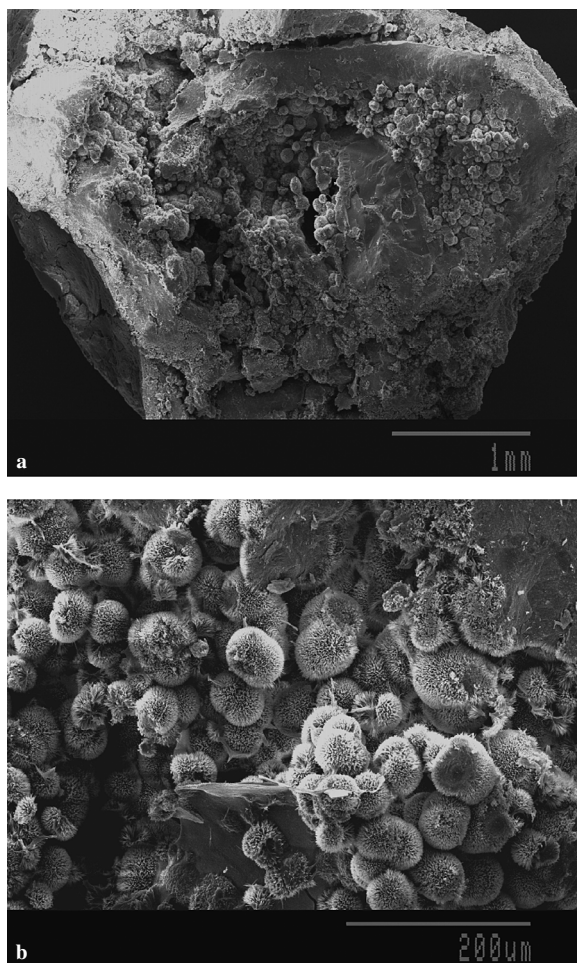


Figure 2.18 Scanning electron microscopy images of an ammonium urate renal calculus: (a) general view of a calculus section; (b) detail where the typical ammonium urate spherulites can be observed.

2.2.6 INFREQUENT RENAL CALCULI

The abundant and continuous intake of some products, for example drugs, can drive continuous urinary elimination of very insoluble substances, that when reaching high supersaturation, can generate renal calculi through some of the described mechanisms. Among the most frequent it is necessary to mention those formed by drugs such as triamterene, indinavir (Figure 2.20), silica, glafenine, sulfonamides, etc. [51].

Other infrequent renal calculi, but not of pharmacological origin, are those of calcium carbonate that are frequent in ruminant animals, and those of calcium urate.

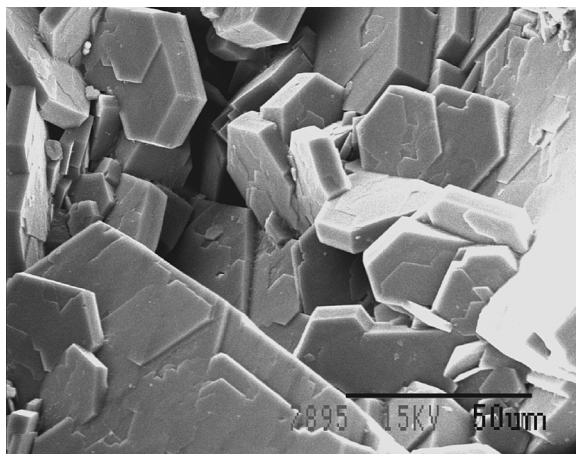


Figure 2.19 Scanning electron microscopy image of a cystine renal calculus. The typical hexagonal morphology of cystine crystals can be observed.

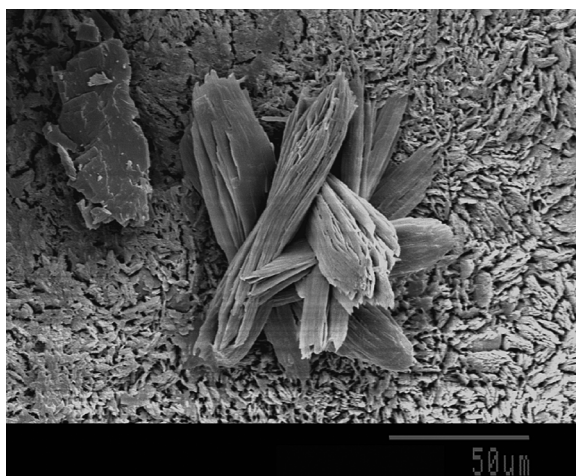


Figure 2.20 Scanning electron microscopy image of indinavir crystals developed on calcium oxalate monohydrate crystals of a calculus formed by a HIV patient treated with this protease inhibitor.

2.2.7 GENERAL CONSIDERATIONS ABOUT RENAL CALCULI

Finally, it should always be kept in mind that renal lithiasis is a multifactorial disease in which diverse factors are implied. Thus, it is a well known fact that not all the individuals with hypercalciuria, hyperuricuria, etc., generate renal calculi.

Also, it should be considered that the specific weight of a certain factor depends on the nature and magnitude of the other factors that participate in the genesis of a certain calculus. For example, if the alterations of the epithelium that covers the papillae are not very severe, they cannot by themselves generate a renal calculus. However, these alterations combined with others can create a favourable situation for the development of a renal calculus. Therefore, in these cases it is evident that the elimination of the second alterations can be enough to avoid the formation of the calculus. For this reason precise knowledge of factors implied in the development of a certain type of renal calculus is so important, it being evident that only from the appropriate study of the renal calculus can a number of possible etiological factors related with its formation be deduced, as can be concluded from Table 2.2. Urine studies can confirm or increase this number of identified lithogen factors, therefore they are obviously also advisable. However, it should be kept in mind that there are alterations which are noticeable only sporadically. When a certain lithogen factor does not appear in a concrete study of urine, it does not mean that it should be discarded definitively, and new studies may be necessary.

2.3 MECHANISMS OF FORMATION OF SIALOLITHS

The macro- and micro-structures of the hydroxyapatite salivary calculi are practically identical to those found in the hydroxyapatite renal calculi (non infective phosphate renal calculi) [22,38]. Thus, the inner fine structure of both types of hydroxyapatite calculi (salivary and renal) is characterized by an ample occurrence of layers of amorphous material, called ‘aspidinic’ hydroxyapatite layers. Aspidinic layers are unstructured from a macroscopic viewpoint (Figure 2.21), but detailed inspection of broken surfaces reveals them to be composed of small spheres of amorphous material cemented together. These stones contain a substantial amount of organic matter both on their outer surface and inside the stone. Hydroxyapatite spheres are largely accumulated in stone cavities as either individual entities or agglomerates (Figure 2.21). The structural similarities between both types of calculus must also involve a similar mechanism of formation. Thus, similarly to the hydroxyapatite renal calculi, small particles of organic matter are the initial substrate on which the calculus development starts. This organic matter is gradually calcified by hydroxyapatite, due to the particular salivary composition and it can be assumed to be later transformed through ageing into an aspidinic structure. The hydroxyapatite crystallization in saliva must be favoured by an appropriate crystallization driving force (thermodynamic factor), i.e. a higher calcium phosphate supersaturation and by a low level of crystallization inhibitors (kinetic factor). Thus, effectively, it was found that the salivary calcium concentration of hydroxyapatite calculi patients was higher than the salivary calcium concentration of the healthy control group [22]. With respect to crystallization inhibitors, it is very interesting to observe how phytate, a potent

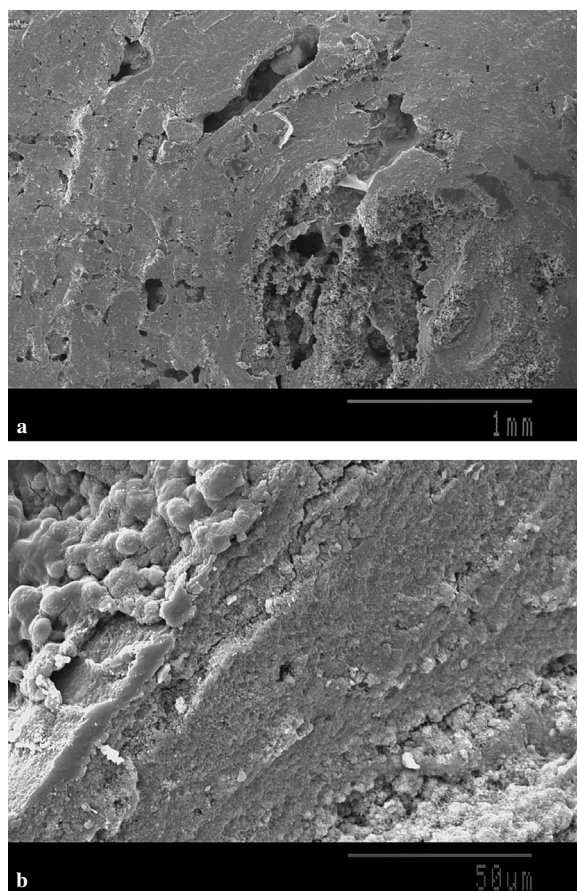


Figure 2.21 Images of a typical salivary calculus obtained by scanning electron microscopy: (a) general view of calculus section; (b) spherulites of hydroxyapatite and aspidinic structures identical to those observed in hydroxyapatite renal calculi.

inhibitor of hydroxyapatite crystallization [52,53], exhibited significantly lower concentrations in saliva of hydroxyapatite stone-formers when compared with the saliva of the healthy control group and also when compared with the saliva of uncalcified (organic matter) calculi patients [22]. Also salivary magnesium concentration (another crystallization inhibitor of hydroxyapatite) of hydroxyapatite stone formers was lower than the salivary magnesium concentration of the healthy control group [22]. Nevertheless, no differences were found in endogenous saliva citrate concentrations between the three studied groups. Citrate has been also described as hydroxyapatite crystallization inhibitor [54], but it must be also considered that citrate can easily increase in saliva due to exogenous contribution through toothpaste, beverages and citrate-rich foods. Obviously, this increase is

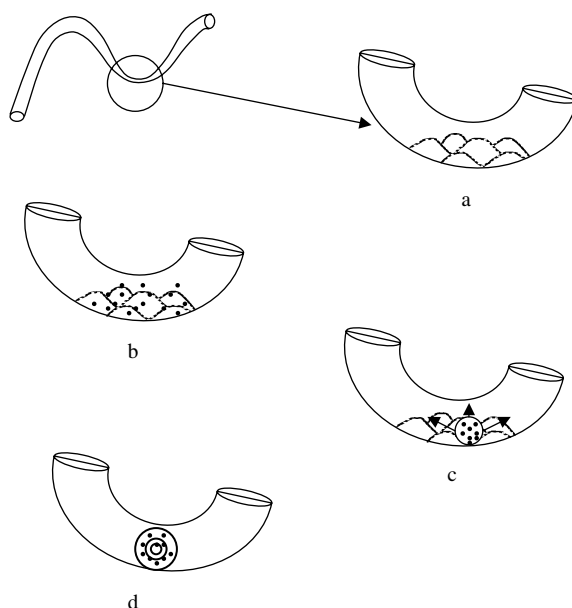


Figure 2.22 Diagram of the mechanism of formation of a sialolith: (a) deposit of organic matter retained in a stenosis of a salivary duct; (b) development of hydroxyapatite crystals into a gelatinous mass; (c) development of the calculus; (d) calculus development ends when the formed concretion totally fills the salivary duct.

only produced in the mouth but not in the salivary ducts. Thus, it can be concluded that the etiologic factors implied in sialolith formation can be classified in two large groups: a) saliva retention due to morphoanatomic factors (salivary duct stenosis, salivary duct diverticuli, etc.) [18,55], and b) saliva composition factors (high supersaturation, crystallization inhibitors deficit, etc.) [54,56,57] (Figure 2.22).

Obviously, the existence of a bacterial infection can favour the development of sialoliths through the increase of salivary pH (this produces an increment of calcium phosphate supersaturation) and due to the increase of organic matter that can obstruct the salivary ducts, favouring the nucleation and retention of hydroxyapatite [58].

As can be seen, an interesting parallelism between the formation of these calculi and renal calculi exists. So, in both cases the presence of retained organic matter, a high hydroxyapatite supersaturation and the deficit of crystallization inhibitors, would permit the development of the first spherulites of hydroxyapatite in the organic matrix, which, by causing a still more effective obstruction of the salivary duct, favours the calculus growth through repetition of the mentioned process. In conclusion it can be stated that the deficit of crystallization inhibitors such as phytate must play an important role in sialolith development.

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3 Calcium and Magnesium Phosphates: Normal and Pathological Mineralization

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3.1 INTRODUCTION

Magnesium and calcium are of utmost importance in biochemical processes in plants and animals. Magnesium ions are mainly incorporated in bone or concentrated inside cells by the action of the membrane, whereas calcium ions are rejected by cells and concentrated in the extracellular fluids [1]. In mammalian skeletal muscle the typical extracellular calcium free ionic concentration is 1.5 mmol L^{-1} while the intracellular concentrations are lower than $10^{-7} \text{ mmol L}^{-1}$ [2]. Magnesium is important in animals to trigger phosphate transfer enzymes, for nerve impulse transmission, muscle contraction and carbohydrate metabolism [3]. Estimations show that more than 90 % of the ATP (adenosine triphosphate) in cells is bound to a magnesium ion, and this metal ion stays bound to the ADP (adenosine diphosphate) formed from the hydrolysis of ATP [2]. Calcium ions are used in the formation of hard tissues, as shells, bones and teeth, as an activator of extracellular enzymes, and in blood clotting. Calcium–magnesium balance is important to trigger the contraction of the muscles, such as those that control the cardiac frequency [4]. Magnesium and potassium metabolism appears to be related, and magnesium deficiency results in myocardial potassium depletion, sodium and calcium influx, and increased vulnerability to cardiac diseases [1].

Normal mineralized tissues contain mainly calcium phosphates with the *apatite* structure, and calcium carbonates. Magnesium ions exist, in normal calcifications, at trace levels inside the calcium-containing solid phase structures and also as

a main component of solid phases, as in whitlockite. Pathological mineralizations have a broader range of mineral phases including the *apatite* group minerals that crystallize under such diverse conditions as inside soft tissues, heart valves, and veins, sometimes causing severe diseases. Brushite, struvite and newberyite can be found in urinary stones, whitlockite is also found in dental calculi, and calcium diphosphate dihydrate is found in pseudo-gout (a gout-like syndrome produced by calcium diphosphate dihydrate crystallization inside cartilage) [5]. Phosphate solid phases containing major cations other than ammonium, calcium, magnesium, potassium and sodium are very unlikely to be found under physiological conditions.

Formation, growth and resorption of the sparingly soluble minerals in *in vivo* environments cannot be explained by the simple transference of the knowledge acquired from *in vitro* experiments. The simplicity of the *in vitro* systems does not account for the complex interactions between the biological material and the inorganic phases. The mineralization processes that occur in organisms are more biologically than chemically driven processes. The right amounts of the mineralizing ions have to be present in the biological system and pass through cell walls to enter the mineralizing environment. Besides the importance of the biological component in the mineralizing/demineralizing processes, the problem of bone calcification, pathological calcifications, and the absorption and excretion of calcium, magnesium and phosphate are intimately connected with the solubilities of the sparingly soluble phosphates. Holt *et al.* [6] had already, by 1925, pointed out that an exact knowledge of the solubility of these salts was important to understand the physiological phenomena and to perform quantitative studies. In order to understand the relationship between the biologically formed solid phases and the composition of the biological fluids in contact with them, it is necessary to have accurate values for the solubility. Brown *et al.* [7] drew attention to the relatively little work done so far to establish the phase equilibria pertaining to the formation conditions of magnesium phosphates. In spite of the large amount of work on calcium phosphates, accurate values for the solubility constants of hydroxyapatite or whitlockite are still needed. Also little is known about the pathogenesis associated with the crystallization of calcium diphosphate in pseudo-gout disease [8].

The formation of soluble metal–ligand ion-pairs or complexes is of vital importance for solubility and the extent of formation of these soluble species, as described by their stability constants, is still unknown for a great number of metals and ligands of biochemical importance. The lack of such formation constants has important implications. The extent of supersaturation of the physiological fluids in relation to the mineralized tissues is difficult to know with any degree of accuracy when it is not possible to know the actual equilibrium concentrations of free metal and phosphate ions in the biological fluids in contact with the solid phases. For the same reason, inhibitory therapy of some pathological mineralizations, associated with some known diseases, is also difficult to promote [9–11].

Phase changes and crystallization or dissolution processes cannot be analyzed only from the thermodynamic view point; it is necessary to take into account the kinetics of the reactions. The latter are specially relevant in the case of calcium phosphates whose different crystal phases have different rates of formation and dissolution.

The degree of super- or undersaturation (S) with respect to a given solid phase is given by the expression

$$S = IP/K_{s0} \quad (3.1)$$

where IP and K_{s0} are the ionic activity product of the solution and the thermodynamic solubility constant, respectively, for the solid phase under consideration. The energy difference needed for any process to proceed (driving force) can be translated by the Gibbs energy change (ΔG) from super- or undersaturated to saturated solutions, defined as

$$\Delta G = -(RT/N) \ln S \quad (3.2)$$

where R is the gas constant, T the thermodynamic temperature and N is the number of ions in the formula unit of the ionic solid [12]. If $\Delta G > 0$ the solution is undersaturated and dissolution rather than crystallization will occur. For $\Delta G = 0$ the solution is saturated and for $\Delta G < 0$ the solution is supersaturated and crystallization can occur. Supersaturation and undersaturation are the physicochemical basis for the formation and dissolution of any solid phase in contact with liquid solutions. Once the value of the solubility constant has been exceeded, the aqueous solution becomes supersaturated and the slow nucleation process can begin. However, supersaturation, by itself, is not enough to promote crystallization since an energy barrier (called 'critical Gibbs energy' ($\Delta_r G^\ddagger$)) has to be overcome to begin the nucleation process. Only when a critical limit of supersaturation is exceeded a fast precipitation of solid phases should occur. The general kinetic aspects of crystallization have to consider the type of nucleation phenomena (homogeneous or heterogeneous), the process of nuclei formation, nucleation rate (number of nuclei formed per unit time per unit volume) and crystal growth rate. When supersaturation increases, the critical Gibbs energy for nucleation ($\Delta_r G^\ddagger$) decreases (energy barrier associated with the formation of critical size nuclei), and the nucleation rate (v_n), at constant temperature, will increase as $v_n \propto \exp(-\Delta_r G^\ddagger)$ [13]. The general expressions that relate $\Delta_r G^\ddagger$ with v_n depend on the crystallizing substances and the type of nucleation phenomena – heterogeneous processes are generally faster than homogeneous.

Dissolution rates are related to undersaturation by the empirical rate expression [14]

$$v_d = k_d(-\sigma)^n \quad (3.3)$$

where v_d represents the dissolution rate, k_d the rate constant, n the effective reaction order and σ the relative under- or supersaturation which can be given by

$$\sigma = (IP/K_{s0})^{1/N} - 1. \quad (3.4)$$

For undersaturated solutions $IP < K_{s0}$ and $\sigma < 0$, saturated solutions have $IP = K_{s0}$ and $\sigma = 0$ and for supersaturated solutions $IP > K_{s0}$ and $\sigma > 0$.

IUPAC [15] nomenclature recommendations are followed in the text. Consequently, mineralogical names will be used only to designate actual minerals and not to define chemical compositions. Mineral names are also used to indicate the structure type and the name of a general group as, for instance the *apatite* group. Synthetic chemical compounds are expressed by its composition following the nomenclature rules. Biomineralization is a natural process of crystallizing minerals and the inorganic materials originated inside or by living structures will be designated by mineral names, whereas the solids synthesized in a laboratory will be designated accordingly their chemical composition. Table 3.1 presents the chemical

Table 3.1 Calcium and magnesium phosphates referred to in this chapter.

Chemical formula	Mineral name	Chemical name	Acronym
CaHPO_4	monetite	calcium hydrogenphosphate	DCPA
$\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$	brushite	calcium hydrogenphosphate dihydrate	DCPD
$\text{Ca}_3(\text{PO}_4)_2$	chloroapatite	tricalcium bis(phosphate)	TCP
$\text{Ca}_5(\text{PO}_4)_3\text{Cl}$		pentacalcium chloride tris(phosphate)	CIAP
$\text{Ca}_5(\text{PO}_4)_3\text{F}$	fluorapatite	pentacalcium fluoride tris(phosphate)	FAP
$\text{Ca}_5(\text{PO}_4)_3(\text{OH})$	hydroxyapatite	pentacalcium hydroxide tris(phosphate)	HAP
$\text{Ca}_{10}(\text{PO}_4, \text{CO}_3)_6(\text{OH}, \text{CO}_3)_2$	dahlite or carbonateapatite		CAP
$\text{Ca}_8(\text{HPO}_4)_2(\text{PO}_4)_4 \cdot 5\text{H}_2\text{O}$		octacalcium bis(hydrogenphosphate) tetrakis(phosphate) pentahydrate	OCP
$\text{Ca}_2\text{P}_2\text{O}_7 \cdot 2\text{H}_2\text{O}$		calcium diphosphate dihydrate	CPPD
$\text{Ca}_9\text{Mg}(\text{HPO}_4)(\text{PO}_4)_6$	whitlockite	nonacalcium magnesium hydrogenphosphate hexaphosphate	TCMP
$\text{MgHPO}_4 \cdot 3\text{H}_2\text{O}$	newberyite	magnesium hydrogenphosphate trihydrate	DMPT
$\text{Mg}_3(\text{PO}_4)_2$	farringtonite	trimagnesium bis(phosphate)	TMP
$\text{Mg}_3(\text{PO}_4)_2 \cdot 8\text{H}_2\text{O}$	bobierite and baricite	trimagnesium bis(phosphate) octahydrate	TMPO
$\text{Mg}_3(\text{PO}_4)_2 \cdot 22\text{H}_2\text{O}$		trimagnesium bis(phosphate) docosahydrate	TMPD
$\text{MgNH}_4\text{PO}_4 \cdot \text{H}_2\text{O}$	dittmarite	ammonium magnesium phosphate monohydrate	AMPM
$\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$	struvite	ammonium magnesium phosphate hexahydrate	AMPH
$\text{KMgPO}_4 \cdot 6\text{H}_2\text{O}$		magnesium potassium phosphate hexahydrate	KMPH

composition, the mineral and chemical names of the solid phosphates discussed in the chapter, as well as the acronyms used to designate the synthetic material.

3.2 STABILITY OF MAGNESIUM AND CALCIUM PHOSPHATES

To establish stability diagrams for the known magnesium and calcium phosphates that occur in normal and pathological biological processes, it is necessary to know the solubility constants of the relevant phases. Struvite, newberyite and whitlockite are the most common magnesium-containing phosphates, and brushite, OCP, *apatite* group solids (hydroxyapatite, carbonateapatite and fluorapatite) and again whitlockite are the most common calcium-containing phosphates reported in the literature in normal and pathological calcifications. Table 3.2 presents the standard Gibbs energies of formation of the biologically relevant calcium and magnesium phosphates, as well as the solid phases with similar stoichiometries that exist as independent phases, and for which experimental solubility constants have been found. The presented solid phases and their standard Gibbs energies of formation are the result of a critical evaluation of the experimental solubility constants [16,17]. The conditions under which the significant phases can occur in biological conditions will be discussed on the basis of the solubility diagram fields presented in Figures 3.1 to 3.4.

The standard Gibbs energies of formation in Table 3.2 were calculated from the relevant thermodynamic solubility constants (K_{s0}) presented in Tables 3.4, 3.5 and 3.6 combined with the standard Gibbs energies of formation given in Table 3.3 through the following expressions

$$\Delta_f G^0 = -RT \ln K_{s0} \quad (3.5)$$

$$\Delta_r G^0(T) = \sum_i \nu_i \Delta_f G^0(X_i, T) \quad (3.6)$$

Table 3.2 Standard Gibbs energies of formation, at 298.15 K, of calcium and magnesium phosphate solid phases.

Solid phase	Stoichiometry	$\Delta_f G^0 / \text{kJ mol}^{-1}$
newberyite or (DMPT)	$\text{MgHPO}_4 \cdot 3\text{H}_2\text{O}$	-2288.94
farringtonite or (TMP)	$\text{Mg}_3(\text{PO}_4)_2$	-3536.54
bobierrite or (TMPO)	$\text{Mg}_3(\text{PO}_4)_2 \cdot 8\text{H}_2\text{O}$	-5443.37
TMPD	$\text{Mg}_3(\text{PO}_4)_2 \cdot 22\text{H}_2\text{O}$	-8752.52
struvite or (AMPH)	$\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$	-3052.27
KMPH	$\text{KMgPO}_4 \cdot 6\text{H}_2\text{O}$	-3240.52
whitlockite or (TCMP)	$\text{Ca}_9\text{Mg}(\text{HPO}_4)(\text{PO}_4)_6$	-13176
monetite or (DCPA)	CaHPO_4	-1681.59
brushite or (DCPD)	$\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$	-2154.84
OCP	$\text{Ca}_8(\text{HPO}_4)_2(\text{PO}_4)_4 \cdot 5\text{H}_2\text{O}$	-12256.8
hydroxyapatite or (HAP)	$\text{Ca}_5(\text{PO}_4)_3(\text{OH})$	-6301.5
chloroapatite or (ClAP)	$\text{Ca}_5(\text{PO}_4)_3\text{Cl}$	-6258.7
fluorapatite or (FAP)	$\text{Ca}_5(\text{PO}_4)_3\text{F}$	-6448.6

^a from the ionic product value presented at 37°C

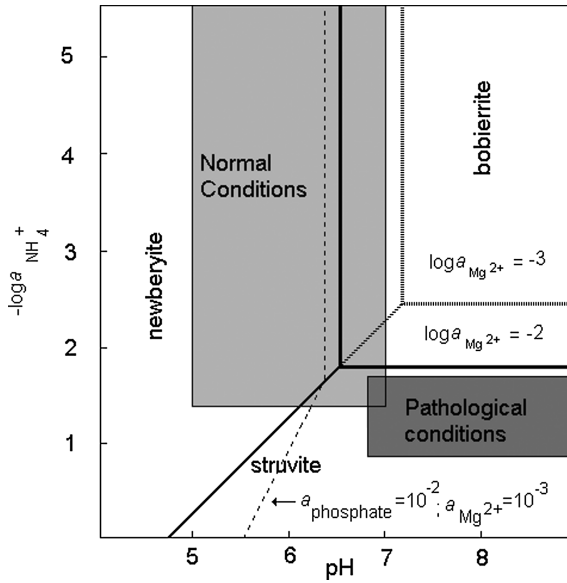


Figure 3.1 Stability field diagram for bobierite, newberyite and struvite, at 298.15 K. Each bold line corresponds to a three phase equilibrium – two adjacent solid phases and aqueous solution. The boundaries in full lines are drawn for $a_{\text{Mg}^{2+}} = 10^{-2}$, the dotted line boundaries are for $a_{\text{Mg}^{2+}} = 10^{-3}$. Dashed lines correspond to the equilibrium of struvite or newberyite with aqueous solutions of total phosphate activity 10^{-2} , and $a_{\text{Mg}^{2+}} = 10^{-3}$.

where ν_i and $\Delta_f G^0(X_i, T)$ are the stoichiometric coefficient and the standard Gibbs energies of formation of species X_i , respectively, for any chemical reaction represented by the equation

$$0 = \sum_i \nu_i X_i. \quad (3.7)$$

Thermodynamic equilibrium constants (K), in general, are defined by the expression

$$K = \prod_i (a_{X_i}^{\text{eq}})^{\nu_i} \quad (3.8)$$

where $a_{X_i}^{\text{eq}}$ is the activity of X_i at equilibrium. The practical relation between the activity of the component X_i and the composition in solution can be defined by Expressions (3.9) or (3.10) according to the basis of the experimental determinations – molality or concentration:

$$a_{m,X} = \gamma_{m,X} m_X / m^0 \quad (3.9)$$

$$a_{c,X} = \gamma_{c,X} c_X / c^0 \quad (3.10)$$

where $a_{m,X}$ is the activity of X on a molality basis, $a_{c,X}$ is the activity of X on a concentration basis, $\gamma_{m,X}$ is the activity coefficient of X on a molality basis, $\gamma_{c,X}$ is the

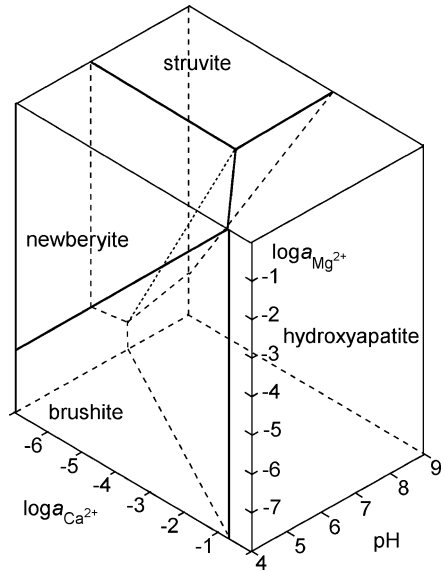


Figure 3.2 Stability field diagrams for brushite, hydroxyapatite, newberyite and struvite, at 298.15 K. Each surface corresponds to a three phase equilibrium – two adjacent solid phases and aqueous solution. The boundary between newberyite and struvite corresponds to $\log a_{\text{NH}_4^+} = -1.5$.

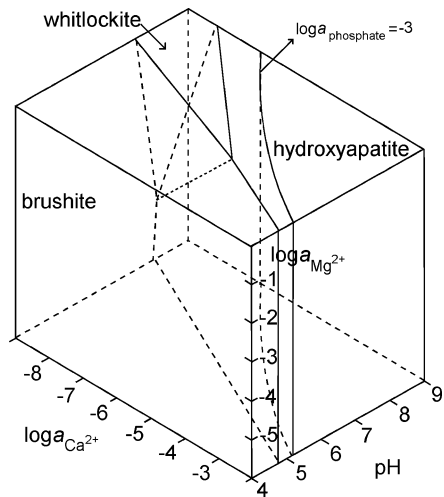


Figure 3.3 Stability field diagrams for brushite, hydroxyapatite and whitlockite, at 298.15 K. Each surface corresponds to a three phase equilibrium – two adjacent solid phases and aqueous solution.

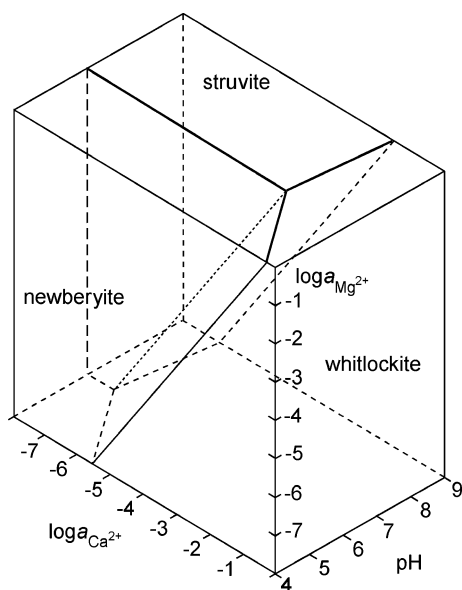


Figure 3.4 Stability field diagram for newberyite, struvite and whitlockite, at 298.15 K. The boundary between struvite and newberyite was established for $\log a_{\text{NH}_4^+} = -1.5$. Each surface corresponds to a three phase equilibrium – two adjacent solid phases and aqueous solution.

Table 3.3 Standard Gibbs energies of formation, at 298.15 K, of relevant species.

Species	$\Delta_f G^0 / \text{kJ mol}^{-1}$	References
$\text{H}_2\text{O}(\text{l})$	-237.141	[19]
$\text{CO}_2(\text{g})$	-394.37	[19]
$\text{Ca}^{2+}(\text{aq})$	-553.5	[19]
$\text{Mg}^{2+}(\text{aq})$	-454.80	[20]
$\text{NH}_4^+(\text{aq})$	-79.37	[20]
$\text{K}^+(\text{aq})$	-283.26	[20]
$\text{F}^-(\text{aq})$	-281.705	[19]
$\text{Cl}^-(\text{aq})$	-131.27	[19]
$\text{OH}^-(\text{aq})$	-157.328	[19]
$\text{PO}_4^{3-}(\text{aq})$	-1019.00	[19]
$\text{HPO}_4^{2-}(\text{aq})$	-1089.50	[19,21]
$\text{H}_2\text{PO}_4^-(\text{aq})$	-1130.61	[19,21]
$\text{H}_3\text{PO}_4^0(\text{aq})$	-1142.88	[19,21]
$\text{P}_2\text{O}_7^{4-}(\text{aq})$	-1919.0	[20]
$\text{HP}_2\text{O}_7^{3-}(\text{aq})$	-1972.2	[20]
$\text{H}_2\text{P}_2\text{O}_7^{2-}(\text{aq})$	-2010.2	[20]
$\text{H}_3\text{P}_2\text{O}_7^-(\text{aq})$	-2023.2	[20]
$\text{H}_4\text{P}_2\text{O}_7^0(\text{aq})$	-2032.0	[20]

Table 3.4 Magnesium phosphates solubility constants, at 298.15 K, taken from Magalhães [16].

Solid phase	Reaction	log <i>K</i>
MgHPO ₄ · 3H ₂ O newberyite or DMPT	$\text{MgHPO}_4 \cdot 3\text{H}_2\text{O(s)} \rightleftharpoons \text{Mg}^{2+}(\text{aq}) + \text{HPO}_4^{2-}(\text{aq}) + 3\text{H}_2\text{O(l)}$	−5.82
Mg ₃ (PO ₄) ₂ farringtonite or TMP	$\text{Mg}_3(\text{PO}_4)_2(\text{s}) \rightleftharpoons 3\text{Mg}^{2+}(\text{aq}) + 2\text{PO}_4^{3-}(\text{aq})$	−23.50
Mg ₃ (PO ₄) ₂ · 8H ₂ O bobierrite or TMPO	$\text{Mg}_3(\text{PO}_4)_2 \cdot 8\text{H}_2\text{O(s)} \rightleftharpoons 3\text{Mg}^{2+}(\text{aq}) + 2\text{PO}_4^{3-}(\text{aq}) + 8\text{H}_2\text{O(l)}$	−25.20
Mg ₃ (PO ₄) ₂ · 22H ₂ O TMPD	$\text{Mg}_3(\text{PO}_4)_2 \cdot 22\text{H}_2\text{O(s)} \rightleftharpoons 3\text{Mg}^{2+}(\text{aq}) + 2\text{PO}_4^{3-}(\text{aq}) + 22\text{H}_2\text{O(l)}$	−23.30
MgNH ₄ PO ₄ · 6H ₂ O struvite or AMPH	$\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O(s)} \rightleftharpoons \text{NH}_4^+(\text{aq}) + \text{Mg}^{2+}(\text{aq}) + \text{PO}_4^{3-}(\text{aq}) + 6\text{H}_2\text{O(l)}$	−13.36
KMgPO ₄ · 6H ₂ O KMPH	$\text{KMgPO}_4 \cdot 6\text{H}_2\text{O(s)} \rightleftharpoons \text{K}^+(\text{aq}) + \text{Mg}^{2+}(\text{aq}) + \text{PO}_4^{3-}(\text{aq}) + 6\text{H}_2\text{O(l)}$	−10.62

Table 3.5 Ionic product for whitlockite.

Solid composition	Reaction	logIP(W)	Reference
Ca ₉ Mg(HPO ₄)(PO ₄) ₆ whitlockite	$\text{Ca}_9\text{Mg}(\text{HPO}_4)(\text{PO}_4)_6(\text{s}) \rightleftharpoons \text{H}^+(\text{aq}) + 9\text{Ca}^{2+}(\text{aq}) + \text{Mg}^{2+}(\text{aq}) + 7\text{PO}_4^{3-}(\text{aq})$	−106.34	[12]

Table 3.6 Calcium phosphates solubility constants, at 298.15 K.

Solid phase	Reaction	log <i>K</i>	References
CaHPO ₄ · 2H ₂ O brushite or DCPD	$\text{CaHPO}_4 \cdot 2\text{H}_2\text{O(s)} \rightleftharpoons \text{Ca}^{2+}(\text{aq}) + \text{HPO}_4^{2-}(\text{aq}) + 2\text{H}_2\text{O(l)}$	−6.58	[17]
CaHPO ₄ monetite or DCPA	$\text{CaHPO}_4(\text{s}) \rightleftharpoons \text{Ca}^{2+}(\text{aq}) + \text{HPO}_4^{2-}(\text{aq})$	−6.76	[17]
Ca ₈ H ₂ (PO ₄) ₆ · 5H ₂ O OCP	$\text{Ca}_8\text{H}_2(\text{PO}_4)_6 \cdot 5\text{H}_2\text{O(s)} \rightleftharpoons 8\text{Ca}^{2+}(\text{aq}) + 6\text{PO}_4^{3-}(\text{aq}) + 2\text{H}^+(\text{aq}) + 5\text{H}_2\text{O(l)}$	−92.7	[37–41]
Ca ₅ (PO ₄) ₃ (OH) hydroxyapatite or HAP	$\text{Ca}_5(\text{PO}_4)_3(\text{OH})(\text{s}) \rightleftharpoons 5\text{Ca}^{2+}(\text{aq}) + 3\text{PO}_4^{3-}(\text{aq}) + \text{OH}^-(\text{aq})$	−56	[17]
Ca ₅ (PO ₄) ₃ Cl chloroapatite or CIAP	$\text{Ca}_5(\text{PO}_4)_3\text{Cl(s)} \rightleftharpoons 5\text{Ca}^{2+}(\text{aq}) + 3\text{PO}_4^{3-}(\text{aq}) + \text{Cl}^-(\text{aq})$	−53.08	[42]
Ca ₅ (PO ₄) ₃ F fluorapatite or FAP	$\text{Ca}_5(\text{PO}_4)_3\text{F(s)} \rightleftharpoons 5\text{Ca}^{2+}(\text{aq}) + 3\text{PO}_4^{3-}(\text{aq}) + \text{F}^-(\text{aq})$	−59.99	[42–45]

activity coefficient of X on a concentration basis, m_X , m^0 , c_X , and c^0 are the molality of X, standard molality, concentration of X and standard concentration, respectively.

Activity coefficients (γ) for the z -valent ionic species X were calculated from the extended form of the Debye–Hückel equation proposed by Davies

$$\log \gamma_z = -Az^2[I^{1/2}/(1 + I^{1/2}) - 0.2I] \quad (3.11)$$

where A is a constant dependent on the solvent and temperature [18], z is the charge number of the ion X , and I is the ionic strength of the solution.

The stability field diagrams used for explaining the crystallization and transformation of calcium and magnesium phosphate solid phases were drawn from values determined at 25 °C. Most of the more accurate existing values were determined for this temperature because the majority of the thermodynamic constants are only known for this temperature. On the other hand, the data obtained by Taylor *et al.* [22] for newberyite and bobierrite, Gregory *et al.* [23], and McDowell *et al.* [24,25] for brushite, monetite and hydroxyapatite, respectively, indicate that the composition of the aqueous solutions change little between 25 and 38 °C, and the experimental errors associated with the values published in the literature are greater than the differences arising from the changes in temperature.

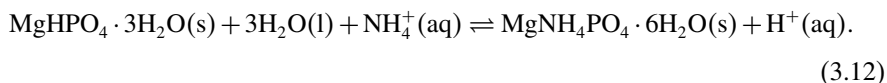
3.2.1 MAGNESIUM PHOSPHATES

Magnesium phosphates as struvite and newberyite are constituents of calculi formed in vertebrates. No reports on the occurrence of bobierrite in vertebrates have been found. The magnesium dihydrogenphosphates are too soluble [7] and it will not be possible to find them under physiological conditions.

To calculate the Gibbs energy of formation of the magnesium phosphates presented in Table 3.2 and to draw the stability field diagrams presented in Figures 3.1, 3.2, and 3.4 the values of the solubility constants from Table 3.4 were used.

The thermodynamic data presented in Table 3.4 report the higher stability of bobierrite (or TMPO) in relation to farringtonite (or TMP) and TMPD that was not yet found as a mineral. Under standard alkaline conditions, at 298.15 K, bobierrite is the thermodynamically more stable phase in the absence of ammonium ions. No report on the occurrence of bobierrite in animal calcifications has been found. Newberyite is found in acidic physiological solutions and struvite is found in more alkaline conditions. Thermodynamic calculations related to possible phase transformations in magnesium phosphates show that newberyite (or DMPT), bobierrite (or TMPO) and struvite (or AMPH) are the most stable magnesium phosphates. A stability field diagram for bobierrite (TMPO), newberyite (DMPT) and struvite (AMPH) is shown in Figure 3.1.

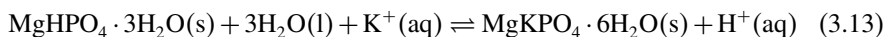
The equilibrium between newberyite (or DMPT) and struvite (or AMPH) is given by



Equilibrium (3.12) shows that the occurrence of struvite and its transformation into newberyite is more dependent on pH and the concentration of ammonium ion than on magnesium and phosphate concentrations.

Hesse and Heimbach [26] reported that the normal urine content in ammonium ion ranges from 0–50 mmol L⁻¹, against 20–120 mmol L⁻¹ in pathological situations, whereas the normal pH is in the range 5–7, increasing to 6.8–8.5 when urinary tract infections are present. These values are represented in Figure 3.1.

Magnesium potassium phosphate hexahydrate with struvite structure (KMPH) is reported by Wang *et al.* [27] in some bladder stones and urinary sediments sampled from buffalo calves fed with high-level cottonseed diet. To analyze the possible conditions of formation of this potassium-containing calculus, the stability field of potassium-containing struvite, at 298.15 K, was defined from the equilibrium boundary of newberyite/KMPH, represented by Equilibrium (3.13) using the thermodynamic data from Tables 3.2 and 3.3.



The equilibrium boundary is given by Equation (3.14)

$$\log a_{\text{K}^+} = 7.55 - \text{pH} \quad (3.14)$$

while the equilibrium boundary of newberyite/struvite, represented by Equilibrium (3.12), calculated at 298.15 K, is given by Equation (3.15)

$$\log a_{\text{NH}_4^+} = 4.81 - \text{pH}. \quad (3.15)$$

Comparing the values of $\log K$ for Expressions (3.14) and (3.15), it is possible to predict that potassium-containing struvite only crystallizes at high values of pH and potassium ion concentration. As for struvite, its crystallization is more dependent on pH and potassium ion concentration than on magnesium and phosphate concentrations.

3.2.2 WHITLOCKITE (TCMP)

Whitlockites and calcium *apatite* group crystals are the main constituents of dental calculi and salivary stones. Whitlockite hexahedrally based crystals, including pseudo-cuboidal and rhombohedral shapes were found in caries-free human tooth enamel, but also crystallized on the tooth surface under tooth calculi and during the enamel caries process [28–30]. Whitlockite deposits occur in physiological or pathological conditions at extra- or intratissular sites as in urinary calculi, aortic valvular tophi, arthritic cartilage and non-infectious diseases of bone tissue [30,31].

Whitlockite is the name of a mineral with the chemical formula $\text{Ca}_9(\text{Mg}, \text{Fe}^{2+})(\text{HPO}_4)(\text{PO}_4)_6$ that may coexist with apatite. It is also the name commonly used to refer to synthetic solids with the chemical formula $\text{Ca}_3(\text{PO}_4)_2$ (TCP). Solids with the latter chemical composition are not found in nature and

can only be obtained by solid-state reactions at temperatures higher than 800 °C. While whitlockite can be found in aqueous environments, at 298.15 K, TCP rapidly dissolves and hydrolyzes in aqueous solutions [6,32]. TCP crystals are isostructural with whitlockite. Crystallographers usually consider whitlockite a magnesium substitute of TCP and discuss the amount of magnesium substitution in the TCP structure. There are different values proposed for the maximum magnesium incorporation in the whitlockite structure that range from 9.5 cation % of magnesium ($\text{Ca}_{19}\text{Mg}_2(\text{PO}_4)_{14}$) to around 16 cation % [33,34] in the synthetic materials. The structure of whitlockite also contains hydrogenphosphate groups in the proportion of one hydrogenphosphate to six phosphate groups [12]. Biological whitlockites contain no iron and slightly lower magnesium in the structure than the maximum magnesium substitution obtained in laboratory synthesis [33], but the majority of the literature describing *in vivo* observations does not report the extent of the magnesium substitution. Whitlockite has similar physical properties to apatite, and Nash [35] states that it is probably misidentified as apatite to explain the lack of references to natural occurrences of the mineral. Scotchford *et al.* [30] call attention to the fact that whitlockite is more observed than is often stated, being most commonly associated with oral sites.

Several researchers [12,30,36] studied the conditions under which whitlockite could precipitate, and observed that seeds of TCMP grew with no significant variation in its composition, *i.e.* with a constant metal/phosphate ratio in the solid, independent of the Mg/Ca and Ca/P ratios in the aqueous solutions, at given values of pH. Crystals of TCMP were obtained, without any HAP and with a chemical composition similar to that proposed for the mineral whitlockite ($\text{Ca}_9\text{Mg}(\text{HPO}_4)(\text{PO}_4)_6$). This is likely to be the most probable composition of TCMP or whitlockite that permits some variations in the Ca/Mg ratio without changes in the structure. Whitlockite forms a small range solid solution series, with magnesium substituting calcium [30]. Whitlockite or TCMP are always used, in this text, to indicate solids with the composition ($\text{Ca}_9\text{Mg}(\text{HPO}_4)(\text{PO}_4)_6$) and not tricalcium bisphosphate (TCP) as it is currently attributed.

No solubility data for whitlockite are available in the literature. To create a possible stability field for this solid phase, the value presented in Table 3.5 for the ionic product (IP(W)) determined by Hamad and Heughebaert [12], under nonequilibrium conditions at 37 °C, and defined as

$$\text{IP(W)} = (a_{\text{Ca}^{2+}})^9 \cdot (a_{\text{Mg}^{2+}}) \cdot (a_{\text{H}^+}) \cdot (a_{\text{PO}_4^{3-}})^7 \quad (3.16)$$

was used to calculate an approximate value for the standard Gibbs energy of formation of whitlockite. The approximate value for the Gibbs energy of formation, presented in Table 3.2 for a solid with the composition ($\text{Ca}_9\text{Mg}(\text{HPO}_4)(\text{PO}_4)_6$) was used to draw the stability field diagrams shown in Figures 3.3 and 3.4.

The calculations of the standard Gibbs energy of formation for whitlockite were done using the standard Gibbs energy of reaction obtained from the IP(W) presented in Table 3.5 by the equation $\Delta_r G^0 = -RT \ln \text{IP(W)}$ where $T = 298.15 \text{ K}$. The use

of the published IP(W) value with a different temperature and without any further corrections results from the uncertainty in the value of IP(W), obtained under nonequilibrium experimental conditions.

3.2.3 CALCIUM PHOSPHATES

Calcium phosphates are the main normal constituents of bones and teeth enamel and dentine. They can also be found in a variety of pathological calcifications as enamel and dentine caries, dental and urinary calculi, salivary stones, soft tissue calcifications, and recently have been widely used as biomaterials for bone implants and in oral medicine. Relevant phases studied in the literature include amorphous calcium phosphates, brushite or DCPD, monetite or DCPA, octacalcium phosphate (OCP), tricalcium bis(phosphate) (TCP) and *apatite* group solids – carbonateapatite (CAP), fluorapatite (FAP) and hydroxyapatite (HAP).

To calculate the Gibbs energy of formation of the calcium phosphates presented in Table 3.2 and to draw the stability field diagrams in Figures 3.2, 3.3, and 3.4 the values of the solubility constants from Table 3.6 were used.

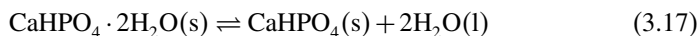
Amorphous Calcium Phosphate

Amorphous calcium phosphate can be formed *in vitro* from highly concentrated solutions of calcium and phosphate ions, at high pH and room temperature. LeGeros [34] and Elliot [46] reported that amorphous calcium phosphates occur as transient phases during the crystallization of other calcium phosphates. Amorphous calcium phosphate is not present in vertebrates' bones but has been found in various pathological tissues of invertebrates [46]. In the presence of aqueous solutions amorphous calcium phosphate transforms, after a few hours, to more stable calcium phosphates according to the pH of the solutions [46]. Transformation to a more crystalline phase can be inhibited by the presence of magnesium, diphosphate and carbonate ions. The transient nature of amorphous calcium phosphate can turn this phase into an important precursor in the crystallization of OCP or HAP in the biological mineralization processes involved in bone calcification and bone implant integration [47].

Calcium Hydrogenphosphates – Brushite (DCPD) and Monetite (DCPA)

Brushite is one of the most important calcium phosphates found in urinary calculi, dental calculi and other pathological calcifications. Monetite has not been found either in normal or pathological calcifications.

The standard Gibbs energy of reaction for the transformation of DCPD into DCPA, represented by Equilibrium (3.17), was calculated using the thermodynamic values from Tables 3.2 and 3.3. The value for $\Delta_r G^0$ is $-1.032 \text{ kJ mol}^{-1}$.



This result shows the possibility of conversion of brushite or DCPD into monetite or DCPA in water, at 298.15 K, under standard conditions, but according to McDowell *et al.* [24], this transformation is accompanied by an 11 % increase in volume. This slightly negative value for the standard Gibbs energy of reaction also shows that monetite or DCPA is slightly more stable than brushite or DCPD under standard conditions. McDowell *et al.* [24] concluded that, at atmospheric pressure, the two salts can coexist at temperatures below 278.15 K, and higher pressures will increase the stability of DCPD relative to that of DCPA. At 310.65 K, monetite is slightly more stable than brushite under standard conditions. In spite of the brushite metastability in relation to monetite the former mineral is more common even in pathological calcifications.

The formation of brushite or DCPD instead of monetite or DCPA, either in nature (with or without the participation of living organisms) or in the laboratory, must be a kinetics driven process. Young and Brown [48] suggested that precipitation of hydrated solids from aqueous solutions is faster than the precipitation of anhydrous solids once hydrated ions are probably more readily incorporated into the crystal structure. On the other hand, hydrated structures must have lower surface energy at the nucleation stage [48], and consequently, lower critical Gibbs energy, requiring a lower degree of supersaturation to nucleate than monetite. The small difference in the solubility of the two solids means that solutions in equilibrium with brushite are not supersaturated enough in relation to monetite to induce its nucleation. Ostwald's empirical rule of states expresses the generalized observations where the first solid phases to precipitate from supersaturated solutions are not the most thermodynamically stable but intermediate metastable phases that slowly transform into the most stable in a stepwise way. The same situation must happen with brushite and monetite and the structural differences between brushite and monetite do not allow a further change of phase.

Octacalcium phosphate (OCP) and Tricalcium Bis(Phosphate) (TCP)

Octacalcium phosphate is a thermodynamically metastable phase in relation to DCPD and HAP and it will change to HAP by hydrolysis at physiological pH [49]. The transition of OCP into HAP can be influenced by the total concentrations of calcium and phosphate in solution, the solution's Ca/P molar ratio, the presence of proteins, and the presence of carbonate, fluoride and magnesium [50,51]. The occurrence of OCP as an intermediate unstable phase during the precipitation of hydroxyapatite is well established [38].

Tricalcium bis(phosphate) is a high temperature solid phase that has not been associated with biological processes. Holt *et al.* [6] stated that this salt hydrolyzes as rapidly as it dissolves. Heughebaert *et al.* [52] and Marques [32] showed that TCP is completely dissolved in less than one day of immersion in aqueous solutions. While TCP is very labile to hydrolysis, not allowing the determination of its solubility by the method of dissolving it in aqueous solution, the metastable OCP lasts for longer periods, and an approximate value for its solubility constant can be determined

(Table 3.6). Owing to these facts no data for the solubility constant of tricalcium phosphate will be presented.

OCP crystallizes in a triclinic lattice but its conversion to the hexagonal HAP is accompanied by small structural changes, keeping HAP the original plate morphology of OCP. The OCP structure consists of two types of alternate layers: an apatite layer (calcium and phosphate ions have similar atomic arrangements to those of apatite) and a hydrate layer. The hydrate layer contains calcium and phosphate ions separated by several water molecules. The *in situ* hydrolysis of OCP to HAP with a small change between the layers can explain the large amount of foreign ions that are introduced into the hydroxyapatite lattice during the ageing process [53]. Calcium amorphous phases, OCP and hydroxyapatites are structures that adsorb or incorporate impurities into their structures [54,55], while brushite and struvite crystallize as pure solids without foreign ions [55]. The structural similarities between OCP and HAP, the easy way HAP grows epitaxially on OCP and the presence of OCP in bones and teeth suggest that OCP is a precursor of HAP in early stages of bone and teeth formation as well as in some pathological calcifications [56]. Once again Ostwald's empirical rule of states can explain this change from a metastable form to the more stable hydroxyapatite.

Apatite Structure Solids

Apatite is the name for a group of minerals with the same crystallographic structure and does not indicate one chemical composition. Stoichiometric hydroxyapatites, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, have been used as prototypes of biological apatites. Their *in vitro* crystallization and behaviour in aqueous solutions under different pressure, temperature and composition conditions have been used to model normal and pathological occurrences, at least in mammals. Solids with this composition have been used as analogs of bones, enamel, dentine and other solid structures to study the mechanisms of dissolution, crystallization, elemental substitutions into the lattice and adsorption properties.

Natural and even the majority of commercial calcium hydroxyapatites are not pure phases but contain different levels of foreign ions such as carbonate, magnesium, aluminium, iron, manganese and zinc [32]. Owing to this fact solubility measurements are usually made with laboratory synthesized solids. The data presented in the literature range from $10^{-42.5}$ to 10^{-66} . The great variability of the solubility data for HAP makes it difficult to choose the best value to calculate a possible stability field for this solid phase. A value of $\log K$ of around -59 for the solubility constant of HAP is the most widely used [14,57], which can be justified by the fact that the peak of the curve of distribution of probabilities for the $\log K$ data is located between -58 and -59 [17]. This value corresponds to a stability for pure hydroxyapatite that suggests some calcium and magnesium phosphates to be stable only under very dissimilar conditions. Following the principle that thermodynamics should be consistent with the actual observations, a value of 10^{-56} for the solubility constant of HAP was used to calculate the approximate value

for its standard Gibbs energy of formation, and to draw the stability field diagram of a solid with the composition $(\text{Ca}_5(\text{PO}_4)_3(\text{OH}))$ presented in Figures 3.2 and 3.3. The standard Gibbs energy for congruent dissolution of a hydroxyapatite with the formula $(\text{Ca}_5(\text{PO}_4)_3(\text{OH}))$, at 298.15 K, is $319.6 \text{ kJ mol}^{-1}$ when $\log K = -56$ and is $336.8 \text{ kJ mol}^{-1}$ when $\log K = -59$. This 5 % difference between the two values for the standard Gibbs energy of reaction is probably lower than the experimental errors associated with the measurements. However, more accurate values for the solubility constants of hydroxyapatite and whitlockite must be found in order to better define the conditions of their stability.

Fluorhydroxyapatites are structurally more stable and much less soluble than pure hydroxyapatites. Fluorhydroxyapatites are more resistant to acid dissolution and the introduction of fluoride ions into dentine and enamel structures has been considered a way to reduce dental caries. The use of fluoride-containing dentifrices is widespread as well as topical applications of fluoride-containing materials [58,59] with positive influence on the dental health of populations. Trace amounts of fluoride are found in normal human calcifications – bones, dentine and enamel (Table 3.7) – and also in human dental calculi [70].

The knowledge of the solubility behaviour of fluorapatite (FAP) is important to understand the mode of action of fluoride ions in inhibiting dental caries [44]. Fluorapatite is more stable than hydroxyapatite [42], and in comparison with HAP, there is a relatively good agreement between the solubility constant data published in the literature for FAP.

Biological apatites are usually carbonate-substituted calcium-deficient hydroxyapatites containing a variety of other minor constituent ions, of which the most abundant are magnesium and sodium, chloride being present in smaller amounts. Biological fluids contain high levels of chloride ions ($\approx 0.1 \text{ mol L}^{-1}$) but low levels of chlorides are found in the structure of bone, dentine and enamel (Table 3.7). The work of Valyasko *et al.* [42] shows that chloroapatites are less stable than hydroxy- and fluorapatites (Table 3.6). In spite of significant amounts of carbonate (3–5 %) always present in bone mineral [14], biological apatites are usually represented by stoichiometric hydroxyapatite neglecting the presence of any foreign ions in the lattice. The substitution of carbonate for phosphate ions in the apatite

Table 3.7 Composition (%) of the inorganic phases of human enamel [194], dentine and bone [34], and stoichiometric hydroxyapatite.

Composition	Enamel	Dentin	Bone	Hydroxyapatite
Calcium, as Ca	36.1–36.5	35.1	34.8	39.8
Phosphate, as P	17.6–17.8	16.9	15.2	18.5
Sodium, as Na	0.5–0.9	0.6	0.9	–
Magnesium, as Mg	0.07–0.44	1.23	0.72	–
Potassium, as K	0.001–0.08	0.05	0.03	–
Carbonate, as CO_3^{2-}	2.05–3.5	5.6	7.4	–
Fluoride, as F^-	0.03–0.34	0.06	0.03	–
Chloride, as Cl^-	0.10–0.65	0.01	0.13	–

lattice results in a change in the crystallographic parameters (it expands in one direction and contracts in another) and morphology, decreases the crystallinity, allows the presence of foreign ions for charge compensation and increases its solubility [14,60]. The stoichiometry of the carbonate-containing apatites is therefore not precisely known, with different extents of carbonate substitutions and the introduction of different ions that can even originate different solid phases. The solubility and dissolution properties of carbonate-substituted apatites are different from those of pure hydroxyapatites and depend also on the extent of carbonate substitution [14]. Carbonateapatites exhibit a range of apparent solubilities – ion activity product at apparent equilibrium – that can probably be correlated with heterogeneities of composition and structure. The dissolved fraction of these heterogeneous solids reaches a ‘stable’ level that reflects more a metastable situation than a true equilibrium [57].

On calculating the solubility constant for a 3.06 % carbonate-substituted hydroxyapatite from experimental measurements, Tang *et al.* [14] omitted the carbonate concentration, and the value for the solubility constant, $\log K_{s0} = -55.77$, was expressed with respect to the stoichiometric hydroxyapatite. The solubility constant of this relatively low carbonate-substituted hydroxyapatite is slightly higher than the value selected in this work for representing the solubility constant of hydroxyapatite, and once again suggests that this value is closer to the actual value than the lowest value of 10^{-59} currently used [14,57].

Stability Field Diagrams

Calcium phosphate stones are more frequently found in cases of hypercalciuria, but solids with the structure and composition of hydroxyapatite are the most stable and widespread calcium phosphates found in soft and hard tissue calcifications related to normal and pathological mineralizations. Associations of hydroxyapatite with struvite are commonly found in urinary calculi when infections of the urinary tract are present. Hesse and Heimbach [26] stated that brushite is formed even in weakly acidic urine (pH 6.5–6.8). At high concentrations of calcium it is expected to find calcium oxalates and brushite in urinary stones, and mixtures of brushite and carbonateapatite are formed for moderate amounts of calcium ($<7 \text{ mmol L}^{-1}$) and pH higher than 6.8 [26].

In order to shed some light on the probable inorganic composition of body fluids that cause the observed calcifications, stability field diagrams for newberyite (DMPT), struvite (AMPH), whitlockite (TCMP), brushite (DCPD) and hydroxyapatite (HAP) were drawn and are shown in Figures 3.2 to 3.4. Using the thermodynamic data from Tables 3.2 and 3.3 the equilibrium boundary surfaces for transitions between brushite and hydroxyapatite, brushite and newberyite, brushite and struvite, hydroxyapatite and newberyite, and hydroxyapatite and struvite were calculated, at 298.15 K, and are shown in Figure 3.2. The boundary between newberyite (or DMPT) and struvite (AMPH) was determined for $\log a_{\text{NH}_4^+} = -1.5$. This value for the ammonium ion activity can be found both in normal and pathological conditions (Figure 3.1).

Brushite was identified by Sanchez-Moral *et al.* [61] in bacterial culture media and remarks were made on the rarity of the situation. Brushite associated with bacterial activity had been reported from Kartchner Caverns in Arizona, USA resulting from decaying of bat guano in an acid-rich environment [61]. Sanchez-Moral *et al.* [61] suggested that brushite from bacterial activity could be related to struvite formation. From Figure 3.2 it is possible to conclude that the rare association of brushite and struvite can only occur at low pH and low magnesium and calcium ion activities.

Association of whitlockite, newberyite and struvite was described by Godinho [33] in a calculus from a human intestine. Hamad and Heughebaert [12] called attention to the fact that whitlockite crystallization takes place under conditions where hydroxyapatite is also a thermodynamic viable phase. This overlap of whitlockite and hydroxyapatite stability fields must be analyzed carefully as it depends on the values considered for the solubility constant of hydroxyapatite. These authors [12] have succeeded in crystallizing TCMP, at 37°C and pH 6.0, within the range of magnesium ion concentrations from 0.290 to 2.510 mmol L⁻¹. This experiment shows that whitlockite must have a narrow stability field, adjacent to brushite and hydroxyapatite, which may explain its rare occurrence in natural systems. The choice of the value for the stability constant of biological hydroxyapatites should consider the possibility of existence of a stability field for whitlockite to explain the existing associations of these two minerals and the stability of whitlockite with respect to apatites. The presence of whitlockite has been discussed, traditionally, only within the perspective of a reactive TCP solid kinetically stabilized by the inhibitory effect of magnesium. Here the possibility of existence of whitlockite as a thermodynamically stable phase will be assumed.

Whitlockite never occurs in its pure state in renal calculi but its presence is detected in mixed forms with calcium oxalate, struvite and *apatite* minerals [62]. Whitlockite is also found associated with hydroxyapatite and OCP in dental calculi [34]. It is not possible to draw a stability field diagram that explains the association of *apatite* minerals, struvite and whitlockite, unless whitlockite has a lower solubility constant value than the one used here. A stability field for whitlockite must exist between struvite and hydroxyapatite and it should appear in the diagram in Figure 3.2. The uncertainty in the stability constants values used for hydroxyapatite and whitlockite is too large and does not allow the construction of a stability field diagram containing all the biologically relevant magnesium and calcium phosphate solid phases. For this reason two stability field diagrams are presented. Figure 3.3 shows a stability field diagram for whitlockite associated with the calcium phosphates brushite and hydroxyapatite, while Figure 3.4 shows a stability field diagram for whitlockite associated with the magnesium phosphates newberyite and struvite considering $\log a_{\text{NH}_4^+} = -1.5$.

The pH and magnesium/calcium ratio in aqueous solutions necessary for *in vitro* precipitation of whitlockite described by Hamad and Heughebaert [12] and LeGeros [34] fall inside the stability field of hydroxyapatite when plotted in Figure 3.3. LeGeros [34] observed that TCMP is resistant to hydrolysis to HAP in contrast to

TCP and OCP that transform to HAP. The stability of whitlockite can be seen as resulting from kinetic inertness, thermodynamic stability or a combination of both. The apparent lack of concordance between the observed experimental results and the predictions that can arise from Figure 3.3 shows again that much more accurate values for the solubility constants of whitlockite and hydroxyapatite are required. Comparison between Figures 3.3 and 3.4 leads to the conclusion that whitlockite either occupies the stability fields of newberyite and struvite or the stability field of hydroxyapatite.

The conversion of DCPD into TCMP mentioned by Hamad and Heughebaert [12], and LeGeros [34] when in presence of magnesium-containing solutions, only can occur if the pH of the solution increases, as can be seen from the stability field diagram presented in Figure 3.3. The presence of magnesium ions in the aqueous solution has no effect on the growth of DCPD and on its ageing process, if the composition of the aqueous solution is maintained within its stability field [55].

3.3 CALCIUM DIPHOSPHATE DIHYDRATE

Calcium diphosphate dihydrate (CPPD) crystals are of particular biological interest due to their association with diseases characterized by acute, subacute or chronic joint inflammation and deposition of these crystals in hyaline cartilage, fibrocartilage and other soft tissues [5]. Crystal deposition may cause bursitis and tendinitis, and may have other consequences such as tendon calcification [63] or destructive arthropathy, a rare but severe complication [64]. CPPD crystal deposition usually involves acute inflammation of large joints like knee, wrist or ankle [65]. Terms like chondrocalcinosis, pseudo-gout or pyrophosphate arthropathy are used to nominate the disease associated with CPPD crystal deposition. Chondrocalcinosis is a general term that is reserved for pathological cartilage calcifications that can include CPPD, brushite, whitlockite, OCP, hydroxyapatite or different combinations of these solid phases [5,66]. Pseudo-gout is often confused with gout because both produce similar symptoms of inflammation characterized by intermittent acute attacks of arthritis [5]. However gout is associated with uric acid salt (monosodium urate) crystallization while pseudo-gout is related to calcium diphosphate dihydrate crystallization. Calcium diphosphate dihydrate, carbonatehydroxyapatite and monosodium urate crystals occasionally coexist in the same articular or periarticular site originating gouty symptoms [64]. Further investigation is needed to understand the significance of such multicrystal coexistence [5].

Despite the biological importance of CPPD, very little is known about the mechanism of formation, growth and dissolution of these crystals [8,67]. The vital process of ATP (adenosine triphosphate) hydrolysis to ADP (adenosine diphosphate) by ATPase enzymes produces inorganic phosphate in plasma, as does the subsequent hydrolysis of ADP to AMP (adenosine monophosphate). However ATP can also be hydrolyzed directly to AMP and inorganic diphosphate, in human plasma, at pH 7.4

by a plasma pyrophosphatase enzyme [68,75]. Inorganic diphosphate can also occur as a by-product of glycosaminoglycan synthesis and other metabolic processes. Hydrolysis of diphosphate to phosphate can take place both in acidic and in basic media [69,75]. At physiological pH the hydrogendiphosphate ion is the main diphosphate species, and it can hydrolyze, at this pH, to the hydrogenphosphate ion (Equilibrium 3.18).



If the hydrogendiphosphate hydrolysis process attained equilibrium, as given by Equilibrium (3.18), the concentration of free hydrogendiphosphate would be lower than $10^{-8} \text{ mol L}^{-1}$ in a plasmatic medium with a free hydrogenphosphate ion concentration of $5 \times 10^{-4} \text{ mol L}^{-1}$, and pH 7.4. The equilibrium constant, $\log K = 8.67$, was calculated for Equilibrium (3.18) from data presented in Table 3.3. The concentrations of inorganic diphosphate ions in normal intra- or extracellular fluids, generally, must be low. Masuda [8] assumes that factors involved in the formation of CPPD crystals should be present in the areas of their formation. According to Christoffersen *et al.* [67] crystals appear to be formed extracellularly in the cartilage matrix, and are usually found close to hypertrophied chondrocytes, which show abnormal metabolism. Crystallization of CPPD must be associated with localized changes, namely an excess production of inorganic diphosphate (PP) due to some alteration of the PP metabolism, as well as changes in the concentration of other solutes and in the tissues of the hypertrophied chondrocytes [75]. Normal chondrocytes in articular and meniscal cartilages generate high concentrations of extracellular inorganic diphosphate that inhibit the physiological calcification of these cartilages, unlike the case of growth plate cartilage [75].

Biochemical studies show that both urinary and plasma concentrations of inorganic diphosphate are normal in patients with pseudo-gout crisis (except those with hypophosphatasia). The average levels of inorganic diphosphate in synovial fluids are 3–15-fold higher than in plasma [71] but the concentrations of inorganic diphosphate increase, specially after a pseudo-gout crisis [5]. Such high levels of inorganic diphosphate do not correlate directly with pseudo-gout, as high levels of these ions can be found in other joint arthropathies [5,71]. However, there is a correlation between joint fluid diphosphate levels and the degree of joint degeneration measured radiologically [5]. The presence of high levels of inorganic diphosphate in joint fluids can be explained by different mechanisms: a defect in the metabolism or production of inorganic diphosphate [72]; liberation of intracellular diphosphate from chondrocytes into the extracellular space; or changes in the concentration of proteoglycan and inhibitory factors [5]. Articular cartilage and other nonmineralized skeletal tissues have high levels of extracellular inorganic diphosphate when compared with mineralized tissues [73].

Although many forms of calcium diphosphate are known (anhydrous forms, three dihydrated polymorphs and other hydrated forms) [60,74], only two polymorphs

of calcium diphosphate have been observed in connection to pseudo-gout, the monoclinic and triclinic forms [60,76], the latter being the dominant one. Crystals size may be up to 10–20 μm in length [77]. No solubility data for CPPD was found in the literature. Wagman *et al.* [20] presented a value for the standard Gibbs energy of formation of $\beta\text{-Ca}_2\text{P}_2\text{O}_7$ that was used to calculate the solubility constant value presented in Table 3.8 for this solid phase.

The values of the solubility constants for the calcium diphosphate hydrated forms should not be very different from the value presented in Table 3.8. Nevertheless, it is not possible to know whether or not the level of CPPD supersaturation in the synovial fluid is sufficiently high to enable local precipitation of this salt. Precipitation may be brought about *in vivo* by localized increases in pyrophosphate concentration generated by cartilage chondrocytes in the neighborhood [8,77]. *In vitro* experiments show that CPPD crystallization occurs when inorganic diphosphate concentrations are abnormally high [60]. During an acute attack of pseudo-gout, CPPD crystals are released from the cartilage cells of the diseased joints, enter the synovial fluid and cause inflammation, possibly due to reactions with immunoglobulins combined with cell rupture. The presence of CPPD crystals in the joints promotes bodily reaction to these foreign bodies, activating chondrocytes and local inflammatory cells. The disease can be idiopathic, secondary or have a genetic tendency [78]. Crystals of calcium diphosphate dihydrate and/or hydroxyapatite are commonly found in older and/or degenerated cartilage like knee joints, while sound articular cartilage matrix does not calcify [8,75]. Idiopathic forms tend to occur in middle-aged and elderly people, the hereditary forms usually appear at relatively early ages [78] and the secondary forms can appear in association with other diseases, like primary hyperparathyroidism, hemochromatosis, hypomagnesemia, hypophosphatasia and surgical intervention of the knee meniscus [5,65,79]. Calcium diphosphate dihydrate deposition may be associated with hypercalcemia in primary hyperparathyroidism, with loss of pyrophosphatase cofactor in hypomagnesemia, inhibition of inorganic pyrophosphatase by iron(II) in hemochromatosis, or with a pyrophosphatase deficiency in hypophosphatasia [5]. Some explanations can be found for CPPD secondary chondrocalcinosis but the pathogenesis associated with primary CPPD crystal deposition in cartilage is not known. In recent years, emphasis has been placed on the importance of age changes in normal cartilages or of local tissue damage related to pre-existing joint diseases that cause abnormalities in the cartilage cells or in the connective tissue [5,8].

Table 3.8 Solubility constant of synthetic anhydrous dicalcium diphosphate ($\text{Ca}_2\text{P}_2\text{O}_7$), at 298.15 K

Solid composition	Reaction	$\log K$	Reference
$\beta\text{-Ca}_2\text{P}_2\text{O}_7$	$\text{Ca}_2\text{P}_2\text{O}_7(\text{s}) \rightleftharpoons 2\text{Ca}^{2+}(\text{aq}) + \text{P}_2\text{O}_7^{4-}(\text{aq})$	-18.54	[20]

3.4 BIOLOGICAL AND *IN VITRO* OCCURRENCES OF CALCIUM AND MAGNESIUM PHOSPHATES

3.4.1 NORMAL CALCIFICATIONS – BONES AND TEETH

Normal calcifications in humans and other vertebrates occur in bones and teeth. Bone is the hard connective tissue that forms the skeleton of vertebrates. It consists of layered organized fibrils of collagen impregnated with calcium phosphates containing carbonates and small amounts of other ions, which compose the mineral part of the bone and constitute 60–70 % of its weight [80]. Bones also contain blood vessels, osteocytes (osteoclasts and osteoblasts) and cartilaginous material. Teeth are hard, conical structures embedded in jaws, composed of three different mineralized structures – enamel, dentine and cementum – that enclose a pulp chamber containing vascularized connective tissue and cells, which forms and nourishes the dentine. Enamel is the most exterior and the hardest material in the crown and covers the dentine. It contains 95–97 wt %, of well crystallized *c*-axis oriented hexagonal prismatic apatite crystals promoted by the ameloblasts [51,80]. Dentin is a bone-like magnesium-rich tissue that forms the bulk of a vertebrate tooth containing around 67 % calcium and magnesium phosphates, the rest being organic material and water. Cementum is the thin layer of calcified tissue that covers the dentine at the root of the teeth and anchors them to the bone.

The inorganic phases present in healthy bones and teeth are idealized as *apatite* structure minerals. The biological apatites of normal body calcifications are nonstoichiometric, having different types and degrees of ion substitution, as is shown in Table 3.7 where the composition of inorganic phases of human enamel [194], dentine and bone [34] is compared with stoichiometric hydroxyapatite. Biological apatites also have different numbers of atomic vacancies, crystallinity and maturation [81]. Biological apatites are carbonate-substituted calcium deficient apatites containing 2–5 wt % carbonate and 5–10 % hydrogenphosphate ion [81,82].

Magnesium is known to inhibit the crystallization of hydroxyapatite and to increase its solubility [34]. Nevertheless, it is present in the composition of all hard tissues, as can be seen from data in Table 3.7, and deposition of cuboid crystals of whitlockite on the surface of functionally normal articular cartilage is frequently observed [83–85]. Scotchford *et al.*, [84,85] reported the presence of whitlockite in all the superficial regions (0–50 μm depth) of the specimens of femoral heads studied, with significantly greater distribution density in the proximal region of the femur heads than in the distal regions, although with less distribution differences in specimens from older patients. The high content of magnesium in dentine may explain reports on the presence of whitlockite in normal teeth. Carbonate ions have a similar effect on apatite, lowering its crystallinity and increasing its solubility. LeGeros [34] suggested that the lower crystallinity and higher solubility of bone and dentine hydroxyapatites compared to those of enamel may be attributed to their higher magnesium and carbonate contents. Data in Table 3.7 also show that despite the

Table 3.9 Range of total concentrations of the inorganic components of human plasma (mmol L⁻¹) [86,87].

Na ⁺	K ⁺	Mg ²⁺	Ca ²⁺	Cl ⁻	SO ₄ ²⁻	Phosphate	Carbonate
142.0	5.0	0.8–1.5	2.1–2.5	103.0	0.5	1.0	27.0

great abundance of chloride ions in biological fluids (Table 3.9) there is a very small incorporation of chloride ions in biological hydroxyapatites. The interconversion of chloroapatite and hydroxyapatite can be represented by Equilibrium (3.19)



whose equilibrium constant has the value $K = 845.7$. Under physiological conditions the ratio Cl/OH in plasma is of the order of magnitude of 10^5 which is much higher than the equilibrium constant, meaning that the chloroapatite phase should be expected as the solid phase present under these conditions. The low substitution of hydroxide ions by chloride ions inside the *apatite* structure can be explained by kinetic constraints, as well as lattice constraints resulting from the differences in ionic radii of hydroxide and chloride.

Blood is a fluid that consists of plasma and cells. The main components of plasma are proteins (mainly albumin, and globulins as lipoproteins and lipid-free proteins) and inorganic ions with the average composition presented in Table 3.9. The plasma lipids, for instance, cholesterol and phospholipids, are almost entirely in the form of lipoproteins that provide a mechanism for the transport of lipids in an aqueous medium. Lipoproteins are possibly involved in the occurrence of atherosclerosis, a deposition of fatty material on the endothelium of arterial walls.

Cells enter the circulatory system from extravascular sites and often leave the blood vessels to enter other extravascular spaces. Under healthy conditions the plasma is in a steady state with the tissue fluids of the body but many biochemical changes occur in disease. The concentration of electrolytes is of utmost importance since they are effective in osmotic pressure control and also aid in the maintenance of the pH of plasma around 7.4. pH is one of the most important factors for *apatite* structure crystallization. The metabolism of bone is important in the regulation of the ionic composition of the body fluids, mainly the calcium content, serving bone as a reservoir for cationic and anionic species [81].

During the formation of teeth and bones, amorphous calcium phosphate, brushite and OCP are considered, by many researchers, as precursors of the crystallization of biological apatites. Also, in the process of bone healing after surgery, trauma or bone implant, and remineralization in carious lesions, brushite and OCP can occur as a transient intermediate in the formation of hydroxyapatite. Crystallization of brushite prior to the appearance of the apatite phase shows that local pH, temperature and plasma composition must differ from homeostatic values, and pH must be lower than 7.4. Schenk *et al.* [88] reported that initial deposition of mineral phase

never occurs in direct contact with the cell surface but always at some distance from it within an organic matrix. According to these authors mineralization must be associated with the presence and removal of mineralization inhibitors as well as with the deposition of cellular derivatives which modify certain matrix components or ionic concentrations in the tissue fluids, and so create conditions which trigger crystal formation. Crystallization of OCP is explained by thermodynamic and kinetic parameters, crystallizing more easily and faster than hydroxyapatite. During the solution-mediated ageing process OCP will turn into hydroxyapatite. Driessens *et al.* [89] concluded that OCP transforms spontaneously into more stable calcium phosphates when in contact with bone extracellular fluid with a composition similar to that presented in Table 3.9. They found that the blood plasma of young humans and other vertebrates had a higher degree of OCP supersaturation than adult species, and that only the blood plasma of healthy human adults was slightly undersaturated with respect to OCP. As OCP has much higher solubility than hydroxyapatite, the blood plasma is always supersaturated in relation to the latter phase. It is important to consider the small differences in blood composition of different vertebrates when experiments are conducted in these animals and the results are extrapolated to humans.

Bone mineralization is a very complex process and several theories exist to explain the mechanisms of calcification. From a chemical viewpoint the mineralization process must have supersaturated solutions, nucleating conditions and growth control developers (inductors and inhibitors). Ossification is a process that is more biologically controlled than chemically driven, but from the chemical viewpoint has to be linked to saturation and supersaturation of solutions. In spite of the relative supersaturation of the plasma in relation to hydroxyapatite, the presence of other etiological factors are fundamental to its crystallization. Bone tissue growth only occurs if osteoblasts (active osteogenic cells) are present. Empirical observations show that the existence of these cells is necessary on the healing bone surface to effect the consolidation of broken bones, as well as an adequate local blood supply. The relative supersaturation of plasma with respect to hydroxyapatite or carbonatehydroxyapatite must not be high enough to promote calcium phosphate precipitation by itself. Enzymes and other calcium binding proteins must be necessary in order to increase the local calcium and phosphate concentrations, and promote the crystallization of calcium phosphates. Nevertheless, proteins, calcium and total phosphate concentrations must be above a certain level to produce proper bone formation.

Nucleation, growth and development of carbonateapatite particles in bone, dentine, cementum and to a lesser extent enamel, are mediated by organic matrix molecules specific for each tissue, notably collagen an important component in all structures except enamel [81]. The matrix is a mixture of soluble and insoluble proteins that works as a compartment, as a possible regulatory system for the whole phenomenon of nucleation and crystal induction, as a regulatory system controlling growth and as a modifier of crystal form [90]. Matrix vesicles in the sites of initial calcification are extracellular particles coated by a membrane, selectively

located within the matrix of bone, cartilage and predentine [91]. They serve as the initial site of calcification in all skeletal tissues [91], and contain a nucleation core of noncrystalline calcium hydrogenphosphate intermixed with calcium binding proteins, and other proteins with high lipid content [92]. Matrix vesicles possess a pH regulation system [93]. This system may function in the matrix vesicle by providing intraluminal buffering capacity. The control of intravesicular pH is important for the stabilization of the nucleation core [93] which, according to Wu *et al.* [92], contains an amorphous calcium phosphate solid phase that turns into *apatite* structure solids as bone matures [94]. When mineralization proceeds, the carbonate substitution in the hydroxyapatite lattice also increases [95]. Miller *et al.* [95] observed that the protein/mineral ratio is higher in younger bones.

3.4.2 PATHOLOGICAL CALCIFICATIONS

While *apatite* structure minerals are the solid phases found in normal mineralized tissues, pathological calcifications contain several solid phases. Whitlockite is found in both normal and osteoarthritic human articular cartilage [83–85], and its frequent occurrence in normal cartilages led Ryan *et al.* [66] to question its pathogenicity in osteoarthritic cartilages. Whitlockite is also found as constituent of salivary stones and in human dental calculi, and infrequently in other pathological tissue calcifications such as abdominal aorta, appendix, lymph nodes of the mesentery, tonsils or certain abnormal calcified cartilages such as intervertebral disks, tracheal cartilage, nasal septum or in urinary stones, and tubercular deposits [30,60,97]. *In vitro* crystallizations of whitlockite, at low temperature from aqueous solutions, show that the major determining factor in the formation of whitlockite are the pH and Mg/Ca ratio in solution. *In vitro* experiments have shown that the presence of carbonate and/or fluoride ions in the solutions favours the formation of *apatite* phase rather than whitlockite [60].

Diseases also arise from hydroxyapatite crystallization in different soft tissues of the body, for instance, veins, muscles and in the extracellular matrix of articular cartilaginous tissues of the joints, causing osteoarthritis. Osteoarthritis is one of the most common disabling disorders, characterized by progressive destruction of articular cartilage, and changes in the calcified cartilage and subchondral bone [98]. The occurrence of nonapatite phases in pathological calcification may indicate that they were crystallized under conditions different from homeostasia or the crystallization of *apatite* structures was inhibited, and less stable phases crystallized instead, without further change to the more stable one. Several authors have stated the compulsory existence of precursor phases, such as OCP, in the formation of bone and teeth [34,99] which change, in time, to the more stable *apatite* structure.

Dental calculi, like some urinary stones and other calcified tissues, are composed of an organic matrix covered by or intermixed with inorganic crystalline phases [34,100,101]. Kido *et al.* [100,102] suggested that urinary stones and human dental calculus may have a matrix with similar components. Calprotectin (calcium binding

protein produced by leukocytes, macrophages and epithelial cells) and osteopontin (calcium binding phosphorylated glycoprotein present in bone matrix) have been found in urinary stones, in human dental calculi and other pathological mineralized body masses, such as arterial plaque, suggesting that these proteins may be involved in their formation [100,102,103], and that the components of the matrix of some of the human body calcifications are similar to those of bone [104]. The layered structure of dental calculi and some urinary stones reflect the different composition of the crystallizing solutions, and the presence or absence of crystallizing nuclei. Diagrams in Figures 3.3 and 3.4 show that slight changes in pH and free magnesium, calcium and phosphate concentrations are enough to promote phase changes under physiological conditions.

Formation of crystals in pathological mineralizations follows the same principles as normal calcifications. Local conditions for nucleation require a certain degree of local supersaturation induced by biochemical processes, which can be promoted by deficiency of inhibitors (like diphosphate, magnesium or even citrate ions) and/or the presence of matrix of organic material (such as cholesterol) or other crystals of different solids, that act as heterogeneous nuclei. The nucleation process is the main step in both normal and pathological calcifications. *In vitro* experiments conducted by Grases and Llobera [105] to simulate the formation of sedimentary urinary stones, demonstrated that in the absence of organic matter no calcium phosphates crystallized in cavities with scarce liquid renovation, but regular hydroxyapatite layers appeared on the wall around the cavity. Visible deposits of calcified organic materials (mixtures of organic matter and spherulites of hydroxyapatite) were formed when a glycoprotein (mucin) was present. In this case, the walls of the cavity as well as the glycoproteins had the capacity to act as heterogeneous nucleants of calcium phosphates. Hydroxyapatite microcrystal nucleation on the surface of epithelial cells can be a critical step in the formation of kidney stones [106] and identical mechanisms can be thought of for calcification in other soft tissues of the body, such as cardiac valves or vascular ducts. Monolayers of hydroxyapatite crystals can bind to epithelial cells. A large amount of kidney stones contain hydroxyapatite as the crystallization nucleus.

A better knowledge of the pathophysiology of different crystal deposits helps in the prevention and treatment of pathological mineralizations. It is well known that extracellular inorganic diphosphate ions can either inhibit or promote different forms of pathogenic calcification [107]. Abnormal cellular production, degradation and transport of inorganic diphosphate ions are associated with specific disease manifestations. Excess diphosphate ions can lead to calcium diphosphate dihydrate crystal deposition originating pseudo-gout, but they are also well known as hydroxyapatite crystallization inhibitors. Hydroxyapatite crystal deposition can result from depressed levels of extracellular inorganic diphosphate ions [107]. LeGeros and LeGeros [60] and Grases *et al.* [10] showed that inorganic diphosphate also inhibits brushite nucleation but the nucleation of hydroxyapatite is more easily inhibited than that of brushite. In fact, inorganic diphosphate has been recognized as an inhibitor of mineralization in connective tissue matrix [73]. The intracellular

calcium concentration is normally low but mitochondria can accumulate, *in vivo*, large amounts of total calcium and phosphate without the formation of any solid calcium phosphates [73].

Abnormal Soft Tissue Mineralization

Phosphate-containing calcifications can be found in a variety of soft tissues such as salivary ducts or glands, tonsils, arterial and venous vein walls, appendix, muscles, cardiac valves, etc. In contrast to whitlockite, the presence of calcium-containing crystals in cartilage is strongly linked to degenerative joint diseases, for instance, calcinosis associated with crystal deposition of the so-called 'basic calcium phosphate' (BCP). It is important to point out that BCP is the denomination given by several authors [65,71,78,96,108] to various mixtures of carbonate-substituted apatite, octacalcium phosphate and tricalcium bisphosphate. As mentioned previously, it is very unlikely to find, under physiological conditions, a compound with the composition $\text{Ca}_3(\text{PO}_4)_2$, therefore the 'basic calcium phosphate' is likely to be a poorly crystallized carbonate-substituted calcium-deficient apatite with different degrees of substitutions, probably intermixed with octacalcium phosphate. Owing to this fact, hydroxyapatite is used as the model compound for many pathological mineralizations of articular cartilage, cardiac valves, etc., but needle-shaped crystals of well-crystallized hydroxyapatite occur in some arthropathies [109]. Best *et al.* [109] report the rare case of hydroxyapatite deposition associated to periarthritis in the temporomandibular joint of a chronic hemodialysis patient. They found relatively uniform density well-crystallized needle-shaped crystals of hydroxyapatite with Ca/P ratio of 1.53, which is very close to the published value of 1.54 for the average atomic ratio of hydroxyapatite in tissue sections [109]. It should be mentioned that hydroxyapatite deposition disease and other single or multiple joint arthropathies are common occurrences associated with chronic renal failure [109–111]. Patients with renal failure, under chronic hemodialysis, have poor control of serum calcium and phosphate concentrations, have hyperuricemia and tendency to develop secondary hyperparathyroidism [109–112]. They display the conditions to develop gout, pseudo-gout and other crystal-induced diseases such as heart, lung and kidney calcifications, as amorphous deposits with compositions approaching whitlockite [111]. Cardiac valve calcification is also frequent in haemodialysis patients, and may contribute to the high cardiovascular mortality observed in these patients [112].

Crystal-induced arthropathy associated with calcium diphosphate dihydrate crystal deposition can occur without any apparent clinical sequelae or in association with osteoarthritis, can cause acute inflammation (pseudo-gout) or occur in association with chronic inflammation resembling rheumatoid arthritis, or with destructive arthritis, where it can be associated with *apatite* structure calcium phosphates [65]. Severe arthropathies with calcium diphosphate dihydrate crystal deposition can occur in tumoral deposits with inflammation and bone destruction, in the wrist with tendon rupture or carpal tunnel syndrome, in the spine

involving ligamenta flava, and in intervertebral discs [65]. *Apatite* structure calcium phosphates can occur almost in the same pathogenesis of degenerative joint disease as CPPD with similar clinical manifestations but are associated directly with calcific periarthritis, tendinitis and arthritis [65,79,108].

The presence of foreign calcium containing crystals, such as *apatite* structure crystals (carbonatehydroxyapatite, octacalcium phosphate) and CPPD in the joints promotes foreign body reactions, activating chondrocytes and local inflammatory cells. The mechanism by which these calcium-containing crystals contribute to joint degradation include induction of mitogenesis in synovial lining cells, stimulation of the synthesis and secretion of cytokines and proteases by phagocytic cells, induction of the synthesis of prostaglandin, as well as phospholipase activation [71,96]. Ryan *et al.* [66] analyzed the impact of partial substitution of calcium by magnesium in these calcium minerals (transforming them into whitlockite), on the above mentioned biological response to the calcium-containing crystals. In spite of the complexity of the processes involved, they concluded that increased magnesium contents of the crystals may induce less severe biological responses. Attempts to dissolve CPPD crystals with the available drugs have been unsuccessful [65,79] but Doherty and Dieppe [113] observed that treatment of chronic CPPD crystal deposition disease with magnesium carbonate decreased, at least, the symptoms without diminishing crystal deposits. Deposits of hydroxyapatite also do not dissolve in response to medication, but in some cases they resolve spontaneously, probably phagocytized by neutrophils or monocytes, where a pH of about 4 is maintained [71,114]. When linked to renal failure, the control of serum phosphate levels may have beneficial effects [79]. Magnesium promotes the dissolution of CPPD and hydroxyapatite crystals [71,96]. The treatment of calcific periarthritis with EDTA sodium salt aqueous solutions is a promising alternative to the classical treatments [78]. Ryan and Cheung [96] suggested that a deeper study of dissolution of the joint-formed crystals by enzymatic methods should be pursued.

Sato *et al.* [98] observed that knee cartilage of osteoarthritic Guinea pigs had higher molar Ca/P ratio than normal animals, suggesting that they had increased carbonate contents in the bone hydroxyapatite. Increased carbonate content is related to changes in the mineral crystallinity during maturation [115]. Sato *et al.* [98] also suggested that osteoarthritic joints have large and matured *apatite* crystals deposited in the calcified cartilage and underlying subchondral bone. These authors also observed that the mineral:matrix ratio was lower in the calcified cartilage and underlying subchondral bone in the osteoarthritic knee joints when compared with the normal joints. The mineral:matrix ratio correlates linearly with the mineral content of the calcified tissues [98]. A lower mineral content may reduce the mechanical stiffness of the calcified cartilage and subchondral bone and presumably increase the vulnerability of these areas to mechanical loads [98].

Sialoliths are calcifications associated with disturbances in salivary gland tissue. The development of sialolith crystals has many origins like disturbances in salivary secretion and changes in the composition of saliva with an increase in salivary viscosity, that cause the formation of microliths and proliferation of bacteria

[116]. Either microliths or bacteria can lead to inflammatory reactions of the tissues with changes in composition and possible accumulation of organic substances, for instance, glycoproteins which promote increases in calcium concentrations and concomitant mineralization of the organic matrix by calcium phosphates [116]. This mechanism is similar to that described for normal calcifications. Some authors [89] suggested that this kind of calcification only occurs as a consequence of local depletion of calcium phosphate inhibitors. An X-ray diffraction analysis of sialolith crystals localized intraglandularly and in Wharton's ducts of the human submandibular gland [116] showed that the nuclei of the sialoliths are mainly carbonateapatites but can also contain whitlockite or, less frequently, brushite. Sialoliths have a layered structure of organic and inorganic substances with the outer shell mostly composed of biochemical molecules and cell detritus. Sialoliths localized in the ducts in the submandibular gland only contain carbonateapatite either in the nuclei or in the cortex shells but those localized in Wharton's ducts have carbonateapatite as well as whitlockite and brushite (only one case reported by Teymoortash *et al.* in 23 analyzed) [116]. The different compositions of the calcifications indicate different compositions of the crystallizing media and the fluids in Wharton's ducts must be richer in magnesium, with higher magnesium/calcium ratios, than ducts in the submandibular gland. Whitlockite only crystallizes from media with small magnesium/calcium ratios as has been reported from *in vitro* experiments. Teymoortash *et al.* [116] made no reference to magnesium, but the fact that saliva is richer in potassium in Wharton's duct than in the intraglandular ducts leads to the conclusion that it must also be richer in magnesium, since both metabolisms appear to be related [1].

Kodaka *et al.* [97] described the composition of five calcifications from different origins (two sialoliths, one urolith, one rhinolith and one tonsillolith) and found biological apatites with sand grain shapes rather than needle-like, plate-shape octacalcium phosphate, and hexahedral crystals of whitlockite precipitated in the internal spaces of the apatite and octacalcium phosphate deposits. All calculi were composed of one or several calcified nuclei covered by thick or thin shells of organic material. The nucleus of the rhinolith was composed only of whitlockite. The porous interior of the tonsillolith was composed of conglomerates containing microorganisms and calcified masses.

The cardiovascular system – heart and blood vessels – is very susceptible to pathological calcifications, the mineral part being constituted mainly by carbonateapatite. Calcification of blood vessels and heart valves is usually an age-dependent disease, but diseases like hypertension or diabetes have a great influence on it. Calcification in the vascular system causes loss of elasticity of the vessels and a reduction in valve performance. Cardiovascular disease due to atherosclerosis is the major cause of mortality and morbidity in the wealthiest countries. Atherosclerosis is a common disease of the arteries, characterized by deposition of plaques in the arterial intima (innermost layer) leading to narrowing of the vessel lumen. Blood vessel walls have three major layers with different thicknesses according to their function, size and location. The outer layer is formed by connective tissue, the

middle is formed by elastic muscular tissue and the intima (endothelium) contains squamous epithelium cells. Vessel calcification with carbonateapatite can occur in the intima and in the middle layers of the vessel walls.

Human atherosclerotic lesions have as their main components cholesterol and calcium which accumulate predominantly within the central core region of the lesions [117]. Sarig *et al.* [117] observed the existence of cholesterol associated with the surface of hydroxyapatite seeds, cholesterol incorporated within calcium phosphate–cholesterol agglomerates produced *in vitro*, and cholesterol within apatite isolated from human atherosclerotic lesions. They concluded that the presence of cholesterol within the centre of calcified granules from atherosclerotic plaque suggests that cholesterol or associated lipids may act to nucleate the deposition of apatite. This association does not occur only in atherosclerotic blood vessels but also in gallstones and calcified cardiac valves. Calcification is a major problem in implanted vascular tissues and is an important cause of bioprosthetic heart valve failure [118,193]. Several mechanisms have been proposed to explain the mineralization of heart valves. The common cause of failure is severe regurgitation due to tear or rupture of one or more mineralized valve cusps [193]. Cell injury as well as its disintegration produces calcification nuclei [193]. Nucleation sites for hydroxyapatite formation can come from either lipoproteins, phospholipids, ions, enzymes released by the dead cells of the transplanted membrane or the lack of calcification inhibitors on the membranes [193]. Banas and Baier [193] proposed that reactive void sites for calcium phosphate mineralization can be originated from structural fatigue damage of the tissue's protein structure. Efforts have been made to enhance tissue valve durability which, in the case of porcine aortic valves, usually lasts between 5 and 15 years [118].

Urinary Tract Stones

The most important phosphates present in urinary tract stone diseases are brushite, carbonatehydroxyapatite and struvite, but it is also possible to find references to whitlockite, hydroxyapatite, octacalcium phosphate (extremely rare), dittmarite (a metastable phase related to struvite) and newberyite [26,101,119,120]. All the apatites present in urinary stones are carbonateapatites, brushite is an acidic phosphate not readily soluble, struvite is the only magnesium phosphate of practical relevance and newberyite is an aging product of struvite [26]. Phosphates are found as the main components in about 12–20 % of all urinary calculi [26,105,119] with an observed downward trend for struvite and an increase in carbonateapatite during the last decade of the 20th century [26,121]. Gault and Chafe [122] and Daudon *et al.* [123] studied altogether more than 43 000 urinary tract stones and concluded that phosphate stones were much less frequent than oxalates but, on average, heavier and more common in women. In general, brushite, carbonateapatite and OCP showed a strong relationship to age with peaks at earlier ages (between the 30s and the 40s), oxalate stones becoming more frequent at older ages.

Thin sections of urinary stones showing the co-existence of *apatite* structure crystals, whitlockite, octacalcium phosphate and struvite can be seen in some

publications [60,101]. Alternate layers of struvite and *apatite* structure crystals were present in several stones, and whitlockite and OCP were found as inclusions inside one of the stones. Hydroxyapatite and organic matter calcified by hydroxyapatite can form the cores of calcium oxalate calculi [101].

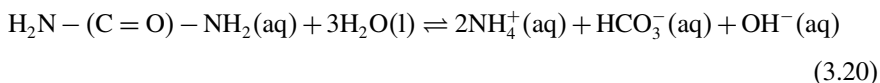
Kohri *et al.* [124] and Marangella *et al.* [125] have found that crystallization of urinary stones could be related to the relative content of magnesium and calcium in water. They reported that the magnesium–calcium ratio of tap water correlated positively with the incidence of struvite stones, and calcium-containing stones had higher incidence for low magnesium–calcium ratios. The magnesium–calcium ratio in tap water depends on regional geology – magnesium–calcium ratios are generally high in regions of basic and sedimentary rocks and low in granite- and limestone-rich regions [124]. Low-calcium diets that can have adverse effects on bone mineralization can be prevented by intake of high-calcium alkaline drinking water, and the urinary stone-forming risk can be decreased by controlling the amount of calcium in drinking water [125].

Struvite

Struvite is found in approximately 16 % of urinary tract calculi [126]. Gault *et al.* [127] and Takasaki *et al.* [128] found that around 50 % of the urinary tract stones contain struvite and half of them are associated with infections. *In vivo* observations and *in vitro* experiments showed that struvite crystallization is, in general, associated with alkaluria (raising of the urine pH) and urinary tract infections by urease-producing bacteria, as *Proteus vulgaris* or *Proteus mirabilis*, *Providencia* spp., *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* [26,129]. Struvite-containing renal stones can be located in the kidney, bladder and ureter [127]. The association of struvite with noxious bacterial activity has special importance for bladder calculi and large renal stones, and has a much lower frequency in ureteral stones [127]. Struvite can also be found in renal carcinoma, and especially in bladder cancer [128]. Thomas *et al.* [130] reported about 12 cases of long ureteral calculi, found in a lumbar or pelvic location, made of a mixture of struvite and carbonateapatite, associated, in most cases, with infection by *Proteus mirabilis*. Grantham and Richmond [131] reported the existence of struvite and apatite in a facet cyst within the lumbar spine of a woman. These authors postulated that there was an infection of the cyst with urease-producing bacteria which caused the crystallization of both minerals within the cyst. McFee and Osborne [132] reported the existence of struvite calculus in the vagina of a female bottlenose dolphin. The occurrence of vaginal calculi in several species of cetaceans is again explained as result of a former (urinary) inflammation with urease-producing bacteria. Struvite also appears related to indwelling catheters and urine drainage system incrustations. Encrusting deposits form on inner and outer surfaces of urinary catheters as a result of infection by urease-producing bacteria. The inner incrustations can lead to catheter blockage in long term urethral catheterization [129,133,134]. These bacteria possess high motility and cover the urine drainage systems with a bacterial biofilm inside which

crystallization occurs. Struvite and carbonateapatite mineralization occurs around the bacterial cells and bacterial footprints have been found by Hesse and Heimbach [26] in the carbonateapatite zones, but not in the struvite zones.

The enzyme urease catalyzes the hydrolysis of urea to hydrogencarbonate and ammonia (Equilibrium (3.20)). The breakdown of urea by urease turns the urine more alkaline ($\text{pH} > 7$) and magnesium ammonium phosphates (struvite) and carbonateapatite can crystallize [26,101,135–139]. Struvite in calculi has to be associated with the presence of ammonium ions in addition to magnesium and phosphate ions under supersaturated conditions.



In vitro studies performed with *Proteus vulgaris* in synthetic urine showed that 60–70 % of the encrusting deposits were struvite and 30–40 % carbonateapatite [137]. Rapid crystallization results in X-shaped struvite crystals whereas slow crystallization (such as that caused by the presence of urease inhibitor) leads to plaques or octahedral crystal habits [138]. In humans, struvite urinary calculi form only when $\text{pH} > 7.0$ and after the onset of a urease-producing bacterial infection [126].

The plastic reconstruction of the bladder (cystoplasty) with intestinal segments has an increased propensity to form urinary calculi, mostly composed of struvite, as compared to reconstruction with gastric segments [140]. Intestinal augmented bladder produces mucus that can act as a possible etiological factor in stone formation [141], and other predisposing risk factors, such as chronic bacteriuria and urinary stasis, can exist [140]. Gastric reservoirs have negligible mucus production and the ability to acidify the urine [140]. Khoury *et al.* [141] observed increased calcium, phosphate and magnesium concentrations and significantly higher Ca/P ratios in the intestinal mucus of stone-forming patients when compared with nonstone-forming patients. These authors suggested measures aimed at clearing mucus from bladder since it appears to have an important role in the genesis of the bladder stones after augmentation. In general gastrocystoplasty remains stone-free, but gastric augmentation stones, when existing, are composed of substances with low solubility in acidic environments such as uric acid [140].

The intestinal mucus is very alkaline, thus the use of intestinal segments in cystoplasty will change the inner conditions of the bladder. The pH of the urine will increase, favouring the crystallization of struvite and even hydroxyapatite or carbonateapatite. Measures to decrease the amount of intestinal mucus in the bladder will help to decrease the value of the pH of the urine and may also decrease the amounts of calcium, magnesium and phosphates in the urine. Supersaturation conditions can be prevented in this way. Gastric segments produce very acidic media and under these conditions the single phosphates that could crystallize would be brushite and newberyite, if supersaturation conditions could be attained.

An acidic urine ($\text{pH} < 6.2$) will dissolve struvite calculi and prevent recurrent struvite stone formation [26]. Nevertheless, struvite can resist mild acidification therapy and can grow in spite of the acidity of the media if it remains within a microbial biofilm where an alkaline pH as well as a magnesium-saturated microenvironment are maintained [139]. To inhibit noninfectious struvite crystallization urinary pH must be ≤ 6.4 . Long term treatment with ammonium chloride can lead to urine pH value as low as 5.5 and, in many cases, reduces the size of the urinary stones and the risk of formation of new stones [26,142].

Experiments with rats have shown that struvite stone formation associated with urinary infections could be prevented by a decrease in urinary magnesium and/or phosphate. Takeuchi *et al.* [143] concluded that calcium- and phosphate-rich diets or low-magnesium diets could decrease urinary magnesium contents and prevent stone formation.

Brushite

Brushite is one of the calcium phosphates present in urinary calculi and is found in around 1 % of the total urinary tract calculi [120,144]. Brushite calculi usually contain numerous cavities partially filled with spherical particles of *apatite* structure crystals and organic matter [101,105,144]. Brushite can exhibit a circular laminar habit formed by compact crystalline layers of plate-like crystals, or aggregates of well developed crystals assembled without any apparent order [101]. Grases *et al.* [101,144] suggested that brushite crystals with the described habits must form by nucleation on the kidney walls or on some equivalent surface, grow by addition of building units from supersaturated urine as a result of low urodynamic efficacy and migrate to the bladder or other urinary system cavities. Brushite is a calcium phosphate that crystallizes under acidic conditions, as can be deduced from the stability field diagrams in Figures 3.2 and 3.3, and usually (in more than 80 % of the cases) it is associated with urinary pH lower than 5.5 [144]. Hesse and Heimbach [26], and Grases *et al.* [144] observed that crystals rarely develop at $\text{pH} > 6.0$ in the cases of slight calcium phosphate supersaturation, which can happen in renal loss of calcium. These conditions favour the association of brushite and hydroxyapatite but do not explain its frequency. Brushite is a very stable phase under the solution composition defined by its stability field. It will hydrolyze to more basic calcium phosphates, OCP and HAP, if the pH of the solution increases and falls out the brushite stability field. A small rise in urine pH can explain the association of both minerals, which can be obtained by dilution of the urine or other small composition changes. Reports on the occurrence of OCP in urinary tract stones are extremely rare but this does not mean that it could not exist as a transient phase during the process of hydrolysis of brushite to hydroxyapatite. The existence of OCP will be a kinetically driven process as hydroxyapatite has a lower crystallization rate than the metastable octacalcium phosphate. OCP will hydrolyze into hydroxyapatite by a solution mediated process. On the other hand, the crystallization of OCP also can be inhibited by some of the urine components.

Brushite calculi formation does not involve urinary tract infection and, if this exists, it is always secondary. These calculi are usually associated with marked hypercalciuria, with excretion values being up to or greater than 10 mmol/day [26], deficit of urinary crystallization inhibitors, hyperuricosuria [144], and some other urinary tract diseases [123]. Brushite crystallization occurs in media with reduced or negligible magnesium content [26,145].

Carbonateapatite

Hesse and Heimbach [26] observed that carbonate is always present in urinary stones with calcium phosphate components and concluded that all the apatites present in urinary stones are carbonateapatites. Carbonateapatite is the most frequent crystalline phosphate phase accounting for around 70 % of the total phosphates [119,144], and is the most frequent inorganic solid phase involved in calcium oxalate urolithiasis, acting as heterogeneous nuclei [10,144]. Incorporation of carbonate ions into the structure of hydroxyapatite occurs when this ion is present in the aqueous solution containing the solid. The amount of carbonate in the *apatite* structure is related to the concentration of carbonate and the pH of the physiological fluids [32]. Carbonateapatites crystallize at pH greater than 6.0, as a primary phase or via phase transformation from brushite or other solids [26,144]. The stability field diagrams for HAP, in Figures 3.2 and 3.3, also include the stability fields for carbonateapatite (or CAP) and OCP. These two phases crystallize under conditions where HAP is the thermodynamically stable phase. The boundary surfaces of CAP with struvite, newberyite and brushite will be parallel to those shown in Figure 3.2 in the direction of diminishing the HAP field and increasing the fields of the other solids. The overall relationship between the phases is maintained. These diagrams can give an insight about the crystallization conditions of some renal stones.

The carbonateapatite proportion in calcium phosphate calculi can be of clinical interest – carbonate rates above 15 % are frequently related to urinary tract infections associated with urease-producing bacteria and rates lower than 10 % are common in cases of carbonateapatite crystallization induced by metabolic disorders. The presence of calcium phosphate calculi containing carbonate in urinary stones is not directly related to a urinary tract infection but infective conditions can favour carbonateapatite formation [146]. Some researchers [119,120] investigated the possible correlations between the composition of urinary stones and aetiological factors. Urinary stones can be found associated with hypercalciuria, hypocitraturia, primary hyperparathyroidism, tubular acidosis, medullary sponge kidney, other urinary diseases and chronic urinary tract infection. Nevertheless, not all people with hypercalciuria, hyperuricosuria, hypocitraturia, etc. generate renal calculi [144]. This is a multifactorial disease, in which one of the factors that can determine the formation of calcium phosphate crystals is the presence or absence of organic matter. Many of the renal calculi contain hydroxyapatite combined with abundant organic matter or grow on an altered epithelial surface [101]. Brushite has been found in hypercalciuric states and primary hyperthyroidism, and whitlockite in urinary

tract infections by nonurease-producing bacteria. Except for struvite, which is the characteristic infection calculus formed as a result of urinary tract infection with urease-producing bacteria, no specific correlations have been found between the phosphate solid phases and nephrolithiasis [26,119].

In Vitro Experiments

Many *in vitro* experiments have been performed during the last century involving synthetic or natural calcium- magnesium- and phosphate-containing materials and/or synthetic or natural urine. In the following we will discuss some studies that illustrate, and are an application of, the models under discussion in the chapter.

To reproduce in the laboratory some conditions prevailing during the formation of urinary stones Grases *et al.* [10,101,105] investigated several synthetic urines with compositions within the range presented in Table 3.10. The *in vitro* inhibitory effect of phytate, diphosphate, triphosphate, medronate, etidronate and others [10], as well as the inductive effect of glycoproteins and other organic matter on the crystallization of brushite and hydroxyapatite have also been studied in order to assess their role in renal lithiasis. Brushite and hydroxyapatite are the most frequent calcium phosphates involved in calcium oxalate urolithiasis, acting as heterogeneous nuclei [10]. The values from Table 3.10 can be used as reference to analyze the composition of urinary stones and its relation to the possible composition of the urinary fluids from which they precipitated.

The Grases *et al.* [145] *in vitro* experiments, with artificial urine containing around 70 mg L⁻¹ of magnesium (similar to the quantity in a normal urine and the value presented in Table 3.10), resulted in the crystallization of brushite accompanied by hydroxyapatite at pH ≤ 7.0 and struvite with hydroxyapatite at pH > 7.0. These results agree with conclusions taken from the stability field diagram in Figure 3.2. This diagram shows that struvite and hydroxyapatite are associated at higher pH than associations of brushite–hydroxyapatite. The values in Table 3.10 represent a possible range of inorganic composition for normal urine. Under normal conditions phosphate ions are present in the urine in high concentrations (approximately ten times higher than those of calcium) and a small rise in pH can cause rapid crystallization of *apatite* structure solids. Comparing the values of the ionic products for newberyite and brushite obtained from the data in Table 3.10, for pH < 6.8, with the stability constants of these solid phases presented in Tables 3.4 and 3.6, a urine with this composition is undersaturated with respect to newberyite but can be saturated with respect to brushite if no calcium complexing

Table 3.10 Composition range of the synthetic urines (mmol L⁻¹) proposed by Grases *et al.* [10,105].

Na ⁺	K ⁺	NH ₄ ⁺	Mg ²⁺	Ca ²⁺	Cl ⁻	SO ₄ ²⁻	Phosphate	Oxalate
159–208	81	43–44	3	3	237–240	17–20	7–35	0.3

agent exists in the normal urine. Brushite crystallization can occur in normal urine. Acidification of urine to $\text{pH} < 6.2$ associated with urine dilution helps in the removal of phosphate stones [26] and also prevents their formation.

Abbona *et al.* [54] studied the *in vitro* precipitation, at 25°C , of calcium and magnesium phosphates in ammoniacal solutions of phosphate, calcium and magnesium in the range of concentrations 0.01 to 0.50 mol dm^{-3} . They observed that pure struvite or newberyite only precipitated from aqueous solutions containing low levels of calcium ($x_{\text{Ca}} < 0.1$) and never precipitated together in the same solution under the same concentrations. Struvite precipitated for pH higher than 5 and newberyite for pH lower than 5.5 , this boundary depending on the total concentration of calcium and magnesium in solution. For a total magnesium concentration of 10 mmol dm^{-3} , only struvite began to precipitate at $\text{pH } 6.8$. Solutions containing $0.1 < x_{\text{Ca}} < 0.7$ at controlled pH precipitated struvite together with amorphous calcium phosphates that changed slowly to *apatite* structure solids. Newberyite was observed only in solutions with magnesium concentrations higher than $0.180 \text{ mol dm}^{-3}$. Calcium has a much greater effect on the crystallization of magnesium phosphates than does magnesium on calcium phosphates. Abbona *et al.* [54] observed that, at 25°C , pure brushite will precipitate even from aqueous solutions containing low levels of calcium ($x_{\text{Ca}} < 0.1$) and also at pH lower than 6.8 (depending on the total concentration of calcium and magnesium in solution). For total calcium and magnesium concentration of 10 mmol dm^{-3} brushite precipitated at $\text{pH } 6.8$ for solutions with $x_{\text{Ca}} = 0.2$, and precipitated in the pH range 5.5 to 6.5 when $x_{\text{Ca}} > 0.7$.

LeGeros and LeGeros [60] reported *in vitro* crystallizations of AMPH (struvite), DCPD (brushite) and TMCP (whitlockite) from aqueous solutions containing ammonium, magnesium, calcium and phosphate ions as a function of the Mg/Ca ratio. When Mg/Ca ratios in solution were $1/0$ or $1/1$ the main product was AMPH, when the Mg/Ca in solution approached $1/1.5$ the main product was DCPD, and when Mg/Ca was $1/4$ the main product was TMCP. Mixtures of HAP and TMCP were obtained from hydrolysis of DCPD in solutions with $\text{Mg/Ca} = 0.2$ and total magnesium concentrations of 2 mmol L^{-1} [34].

It is believed that, during bone development and other calcification processes, osteopontin (a phosphorylated glycoprotein) secreted by osteoblasts to the mineralizing extracellular matrix facilitates the attachment of the osteoblasts and osteoclasts to that matrix, allowing them to perform their functions during osteogenesis [103] or hydroxyapatite crystallization. Lieske *et al.* [106] performed *in vitro* studies to inhibit the adhesion of hydroxyapatite to the apical surface of renal tubular cells, by diverse polycations and polyanions including many found in tubular fluids such as chondroitin sulphates A and B, citrate, nephrocalcin or osteopontin. Osteopontin like other proteins can participate in processes of hydroxyapatite crystallization. Some authors [105,106,147] concluded that the inhibition or promotion of the crystallization by substances like osteopontin, phytate or citrate depends on specific conditions of the cell environment such as types of cells, presence or absence of given substances, concentrations, etc.

Dental Calculi and Dental Caries

Brushite, whitlockite, OCP or carbonatehydroxyapatite are present in human dental calculi [28–30,148,149]. Dental calculi can be seen as the calcification of the dental plaque biofilm, composed of calcium-containing phosphates, deposited between and within remnants of formerly viable microorganisms, where inorganic ions are provided by saliva or crevicular fluids [150]. Human dental calculi are formed by an organic and an inorganic phase whose compositions depend on age (personal and of the calculus), location (tooth, supra- or subgingival), depth within the calculus, or whether in contact with saliva or the different tooth mineralized structures [34,148,150,151]. Barrea *et al.* [151] observed that supragingival calculi (usually found at the exits of the salivary ducts [148]) are composed mainly of carbonatehydroxyapatite while calculi from subgingival regions accumulate magnesium, being mixtures of different calcium phosphates. A high resolution electron microscopy study of the junction between a newly formed dental calculus and the enamel surface revealed that lattice fringes of *apatite* structure dental calculus crystals directly coincided with those of enamel crystals [148,152]. The contact of the crystals of the dental calculus with the enamel crystals could be either side by side or tip to tip [152]. Hayashi [152] suggested that the elongation and/or enlargement of the lattice fringes of calculus crystals during their growth bring direct coherence with enamel crystals. According to this author, this fact can explain the clinical difficulty in completely removing dental calculi without the loss of the superficial layer of enamel. Dental calculus can also be found in the tooth pulp, usually associated with infections.

Fluoride distribution, in human dental calculi from different countries and different sites on the tooth surface, was studied by Okumura *et al.* [70] and Huang *et al.* [153,154]. Calculi with the highest fluoride content were recorded from populations living in fluoridated areas [154] but no correlation was established between the calculus' fluoride concentration and the different fluoride amounts in the environment. Okumura *et al.* [70] and Huang *et al.* [153] found that the highest fluoride amounts were at the outer surface of the dental calculus, falling off to a plateau for the interior of the calculus, and rising again as the tooth surface was approached. The different fluoride concentrations at different parts of dental calculus are probably due to different composition and mechanism of formation [153]. Fluoride is associated with hydroxyapatite, as brushite does not incorporate foreign ions in its structure [55] and the crystallization of whitlockite is inhibited by the presence of fluoride. Hydroxyapatites that are formed via OCP as a precursor probably contain higher levels of foreign ions since the open structure of OCP accommodates larger amounts [34].

Hayashi [155] investigated the initial stage of a dental calculus on enamel surface and observed the existence of a thin layer of needle-like hydroxyapatite crystals. These observations indicate that the local environment has the conditions to produce this solid phase and the growth of calculus crystals must advance through additional mineralization [155]. On the other hand, brushite is a common phase in deposits of calculus around the teeth and appears to be the first phase to crystallize during the formation of human dental calculi, with further change to OCP that seems to

be a rather long lasting phase [28,34,116,148]. Crystallization of brushite can only occur under acidic conditions that can be attained locally by the kind of food and drink, by bacterial processing or by changes in saliva itself. These observations lead to the conclusion that acidic mouth conditions promote the formation of dental calculi containing brushite associated with OCP and hydroxyapatite. Small increases in the saliva pH will promote the hydrolysis of brushite and its change to OCP and later to hydroxyapatite. Kodaka *et al.* [28] applied resin plates to the lingual sides of the mandibular gingival regions in eight humans and studied the mineral deposits formed on them. They found that calcium deficient apatites associated with transitional forms between hydroxyapatite and OCP crystallized in all subjects. Brushite crystallized in half of the subjects and crystals with a shape and Ca/P molar ratio similar to OCP were observed in three subjects. Magnesium containing apatites were found in two subjects.

Whitlockite can coexist with OCP and hydroxyapatite in dental calculus samples, in inner and outer layers, on the enamel and cementum surfaces, as well as on the cervical enamel surface and/or on the root surface [60,148,156–158]. Hayashi [158] observed that the mineral deposits crystallized on a thin adhering pellicle formed over an intact tooth enamel surface were composed of whitlockite and aggregation of fine hydroxyapatite crystallites. This author found that the observed deposits were in a free floating condition without relation to the enamel surface and that whitlockite and hydroxyapatite can exist independently and stably *in vivo* [158]. Whitlockite crystals can also be found in caries-free old human tooth enamel crevices as tufts and lamellae, to a lesser degree in the inner crevices of caries-free exfoliated deciduous enamel, and not at all in sound young enamel [29]. Kodaka *et al.* [29] suggested that whitlockite will grow over a long period after eruption of the tooth or during the enamel caries process. The coexistence of several calcium and magnesium-containing phosphates can be explained by the existence of specific sets of conditions (temperature, pH, composition of the solution (Figure 3.3)) that allow the crystallization of a given solid phase and its change to other solid phases. Under the right conditions changes between these three solid phases should be fast, as the three phases have similar *apatite* based structures. LeGeros *et al.* [148,159] suggested that the crystallization of whitlockite in human dental calculi is favoured by the Ca/Mg ratio in conjunction with the pH of the human saliva. A higher value of pH (pH 8.5) for dogs' saliva promotes the formation of calcium carbonates instead of the phosphates observed in human oral calculi [148,159].

The presence of different phases is related to the composition of the fluids in contact with them but no significant differences were found by Pattanaporn and Navia [160] in saliva flow rate, pH or buffer capacity between people with and without dental calculi. Saliva and the other body fluids are supersaturated with respect to hydroxyapatite, and under given conditions can also be saturated with respect to other calcium phosphates, such as OCP and whitlockite [161]. Nevertheless, supersaturation by itself does not explain stone formation. Once more, this is a biologically driven process with local increases of calcium and phosphate ions promoted by biochemical polymeric molecules capable of inducing solid

nucleation. Some salivary proteins adsorb on the tooth surface and are essential for microbial adherence [149]. Microorganisms then induce the formation of stones either by promoting the right environment for homogeneous nucleation or inducing heterogeneous nucleation by their remnants [148,162]. Acidic phospholipids and proteolipids present in cell membranes help in microbial induced mineralization [149]. Van Dijk *et al.* [163] isolated a calcium-precipitating proteolipid involved in the bacterial formation of a hydroxyapatite-containing dental calculus.

Apatites (hydroxy-, fluor-, carbonate-), whitlockite and OCP as well as fluorite (calcium fluoride) have been associated with the caries process of enamel and dentine, a bacterially based disease [34,164]. The caries process can have several stages (initiation, progression, and arrest) resulting in the formation of lesions that can cross the tooth's layers. Enamel caries begin with a subsurface acidic demineralization that can be reversible in the early stages while the surface remains intact [82,165]. The reverse process of crystallization can be accelerated by the presence of fluoride. Caries progression is associated with pathological factors such as the action of acidogenic bacteria, salivary dysfunction or dietary fermentable carbohydrates [164]. The acid produced by bacterial action diffuses into the tooth and dissolves the carbonatehydroxyapatite [164]. The preferential loss of carbonate over phosphate is noticed during the demineralization process [34,82] which can be explained by a surface change of the carbonatehydroxyapatite into solid phases containing, globally, higher amounts of hydrogenphosphate and fluoride ions, lower amounts of magnesium ions and lower Ca/P ratios [34]. Microscopic analysis of dentine caries show associations of well crystallized hydroxyapatite and whitlockite but the latter has not been identified in enamel caries [34]. LeGeros [34] explained this fact by the higher magnesium content of dentine in relation to enamel (Table 3.7) causing a higher Mg/Ca molar ratio in the localized microenvironment resulting from the solid dissolution, and creating favourable conditions for whitlockite formation.

Saliva plays a crucial role in preventing dental caries, by maintaining the plaque pH and controlling the enamel de- and remineralization processes [166]. In normal saliva the presence of acidic proteins, diphosphate ions as well as other low and high molecular weight saliva species are considered to be the main promoters and/or inhibitors of spontaneous crystallization of *apatite* crystals from saliva [34,167]. According to White and Cox [167] this dual role simulates the reactivity of similar macromolecules in controlling bone and tooth formation. The inhibitory effect of diphosphate, magnesium and zinc ions, and some organic molecules for the prevention of dental calculi is similar to the inhibition of calcium phosphate mineralizations [34,151,162,167–169]. However, excessive concentrations of some of these elements in saliva can promote the crystallization of other solid phases, and also their incorporation into the crystalline structure of calcium phosphates [34,192]. White and Cox [167] suggested that the major limitation of salivary macromolecules in controlling plaque mineralization arises from their slow diffusivity into plaque prohibiting them from controlling localized increases in supersaturation. Salivary calcium, phosphate and proteins, fluoride and antibacterial agents can balance, prevent or reverse dental caries [164]. Some of

the inhibitory agents are introduced in toothpaste to control calculus formation and diphosphate-containing dentifrices significantly inhibit calculus formation [170]. To prevent or repair incipient dental caries and to help the remineralization process, some authors [166,171] suggested that soluble amorphous calcium phosphate, fluoride and hydrogencarbonate compounds could be used as additives in dentifrices, mouthrinse solutions, aerosols and even chewing gums. When applied, they release calcium, phosphate, fluoride and hydrogencarbonate ions that regulate pH, and promote precipitation of *apatite* structure solids owing to an increase of calcium and phosphate ions in the saliva.

Gallstones

Gallstones are nodules formed within the gallbladder and, with much less incidence, in biliary ducts within or outside the liver. Cholesterol, bile acids and calcium are the major constituents of gallstones, with iron, phosphates, carbonates, proteins, carbohydrates mucus and cellular debris being other constituents of these stones [172,173]. The majority of gallstones are composed of cholesterol, but calcium present as inorganic or organic salts (carbonate, phosphate, bilirubinate, palmitate) is also an important constituent [87,173]. The typical cholesterol stone, with small amounts of calcium salts within the core or peripheral shells of calcium carbonate, is usually white to yellow in colour. Brown stones contain calcium bilirubinate, small amounts of cholesterol and always calcium palmitate, whereas black stones contain mainly bilirubin polymers, calcium bilirubinate, calcium carbonate and/or phosphate and seldom cholesterol and calcium palmitate [175–177]. Hydroxyapatite can be a significant component of black pigment gallstones [176,178] that constitute around 10 % of total gallstones and 60 % of the pigmented [175,177].

Calcium ions are introduced in bile during the early stages of its formation by pathways that involve direct or indirect contact with the blood serum [87]. Rudnicki *et al.* [87] analyzed human blood serum (plasma) and gallbladder bile, and the composition of the inorganic components determined are presented in Table 3.11, as well as the values presented by Ohtsuki *et al.* [86]. Human bile and plasma contain substances that complex with calcium and interfere with the precipitation of calcium phosphate and carbonate. Driessens *et al.*, [89,179] found that, under normal conditions, 45 % of the total calcium in the plasma of man and other mammals is in the ionized free form. Taking this into account as well as the ionic strength of both media, Rudnicki *et al.* [87] proposed the free equilibrium calcium ion concentrations ($[Ca^{2+}]_{eq}$) presented in Table 3.11. Values of phosphate presented in this table correspond to the total free phosphates existing in each media.

The calcium phosphates found in gallstones are mostly hydroxyapatite which can be explained by the pH of the bile and its inorganic and organic composition [180]. The lithogenic potential of bile depends not only on its supersaturation in relation to the components of the solid phases but also on the presence of nucleating inductors or inhibitors. Mucin (a mucous glycoprotein) is the main secretory product of gallbladder epithelial cells and its presence in the gallbladder

Table 3.11 Range of total (T) and equilibrium (eq) concentrations of the inorganic components of human serum and gallbladder bile (mmol L⁻¹) [86,87].

medium	[Na ⁺] _T	[K ⁺] _T	[Mg ²⁺] _T	[Ca ²⁺] _T	[Ca ²⁺] _{eq}	[PO ₄ ³⁻] _T
serum	142.0	5.0	0.8–1.5	2.1–2.5	1.2	1.0
bile	142–200	5–11	1–5	2–7	0.4	0.3–1.3

bile promotes gallstone formation (mucin forms the structural matrix of stones) [181]. Increased gallbladder mucus secretion has been implicated in gallstone formation in humans. The mechanism underlying control of mucin synthesis and secretion by the gallbladder is not known. Grases and Llobera [105] observed that mucin increases the crystallization of brushite and hydroxyapatite when present in synthetic urine. Qiu *et al.* [182] also observed that mucin promotes the crystallization of hydroxyapatite but they concluded that this ability could be altered by pathological states, like bacterial infection, that could cause the digestion of mucin. Bile acids (cholic, deoxycholic, lithocholic, glycocholic, etc.) produced in the liver result from cholesterol biosynthesis. The main characteristic of bile acids is the carboxylic group in their five-carbon side chain, which in many vertebrates is bound, in the liver, with glycine and taurine by a peptide linkage [172]. Glycine-conjugated bile acids bind strongly on the calcium phosphate surface, inhibiting either the transition of amorphous calcium phosphates into crystallized hydroxyapatite and the consequent crystal growth [178,180], or the crystallization of the hydroxyapatite itself [180,183,184]. The taurine-conjugated bile acids do not affect the crystallization of hydroxyapatite [178] but prevent the formation of solid amorphous calcium phosphate by lowering the free calcium ion activity in the solution [185]. These observations lead to the conclusion that the extent of calcium complexation with glycine-conjugated bile acids is much higher than with taurine-conjugated bile acids. The former lowers the activity of free calcium ions much more and prevents, in some cases, the nucleation and growth of calcium phosphates [180].

Normal bile has pH > 7.10 and Rudnicki *et al.* [87] reported a value of 7.25 for the pH of normal bile and 6.94 for the pH of stone-containing bile. For all the reported pHs and the calcium and phosphate concentrations presented in Table 3.11 the thermodynamically stable phase is hydroxyapatite. The presence of calcium in bile in large amounts does not mean that under normal health conditions it will be free for gallstone nuclei formation, since it binds strongly to many of the bile components [186]. The formation of hydroxyapatite in gallstones results from a subtle balance of interactions which, on one hand, keeps the isolated calcium ions strongly bound to bile components, as glycine-conjugated bile acids, and on the other hand promotes the reaction of calcium and phosphate ions with cholesterol or mucin and other glycoproteins, resulting in the possibility of formation of more or less crystalline calcium phosphate nuclei that will evolve into hydroxyapatite. It has been suggested that calcium salt precipitation is a requisite for gallstone formation and pigmentation [172].

The formation of gallstones is associated with different disorders and some relations can be established between the composition of gallstones and aetiological factors [176,177]. Kaufman *et al.* [177] concluded that the different gallstone aetiologies are reflected more in their peripheral compositions than in their central regions. Cholesterol and pigmented gallstones differentiate more during the growth process than in the nucleation process [177]. Cholesterol stones are invariably associated with gallbladder hypomotility and/or with hypersecretion of cholesterol [176]. Black pigment gallstones are mostly found in the gallbladder, and can result from hepatic hypersecretion of bilirubin conjugates associated with liver damage (hemolysis), possibly of enterohepatic cycling of unconjugated bilirubin in nonhemolytic states and of hyperplastic inflammation of the gallbladder (cholecystosis), but it is uncommon to find black stones linked to gallbladder hypomotility [175,176]. It is also very unlikely to find black pigment gallstones associated with abnormal bile production, or with bile salt hyposecretion, as can happen in cirrhosis. Bile salt deficiency causes incomplete solubilization of unconjugated bilirubin and weak binding of calcium ions [176]. Prolonged retention of bile in the gallbladder as a result of poor biliary drainage (stasis) and anaerobical bacterial infection is responsible for brown pigment gallstones being formed, usually in the bile ducts [176]. Kodaka *et al.* [187] described cholesterol rich gallstones containing no calcium salts or containing peripheral concentric layers of calcium carbonate or calcium phosphate. The calcium phosphate-containing stones had no calcium salt cores. The cholesterol poor stones showed dispersed particles either of calcium phosphate or mixtures of calcium phosphate with bilirubinate and/or palmitate.

Calcium carbonate with *calcite* structure is the major constituent in gallstones, being present in around 50 % of the cases examined by Manoli and Dalas [188], but aragonite and especially vaterite are found as well. Total concentration of carbonates in bile juices is also high in gallstone-containing individuals when compared with normal subjects [174] which must promote the formation of carbonateapatite instead of hydroxyapatite as is reported in the literature. In fact, there are many references to the coexistence of calcium carbonates and calcium phosphates in the same gallstones but the classification of the calcium phosphates is, in many cases, lacking. It is possible that the frequent denomination of ‘amorphous calcium phosphates’ refers to a very low crystalline carbonateapatite containing high levels of carbonate that is known to decrease the crystallinity of the *apatite* structure.

3.4.3 CALCIUM PHOSPHATE BIOMATERIALS

Calcium phosphate biomaterials are used for bone repair and in restorative dentistry. Commercial calcium phosphate biomaterials are either single phases (hydroxyapatite, β -tricalcium phosphate and calcium hydrogenphosphate) or mixtures of HAP and TCP or HAP and DCPD. They are available as ceramics, metal coatings, cements and polymer composites. One aim of the biomaterials is to

promote osteogenesis and in this respect HAP has a similar structure to the mineral constituents of bones and teeth and is largely used in orthopedics and dentistry to improve bone–implant links in order to stabilize the metal implant. It is also used as filler for the repair of bone defects or bone loss. In general the implanted material dissolves and recrystallizes with a structure similar to the bone, as a result of the interactions of the implanted calcium phosphates and the body fluids mediated by the action of the osteoblasts.

In vitro experiments show that synthetic hydroxyapatite changes to a solid phase with a composition approaching that of bone when ageing in aqueous solutions with similar composition to that presented in Table 3.9 for body fluids [189]. A large amount of work has been published, since the beginning of the 20th century, on *in vitro* formation, transformation of calcium-containing phosphates and the action of inhibitors of crystallization, in order to understand the *in vivo* processes of normal and pathological mineralizations, of bone and teeth demineralization and also the osteointegration of bone implants.

In vivo experiments show that autogenous cancellous bone chips promote osteointegration faster than synthetic hydroxyapatite [190]. Experiments with calcium phosphates show that amorphous hydroxyapatite undergoes distinct bulk degradation while in the highly crystalline hydroxyapatite and whitlockite, degradation was negligible after four weeks of implantation in rat femora [191]. The presence of high levels of magnesium ions, as in the structure of whitlockite, seems to inhibit the degradation processes. Less crystalline calcium phosphates seem to be beneficial over highly crystalline bone coatings or implant materials and their similarity to bone inorganic structure improves the bone formation and osteointegration.

ACKNOWLEDGEMENTS

To José Alberto L. Costa for helping with the computer drawing of the four figures presented in this work.

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4 Relevance of a Polymer-Induced Liquid-Precursor (PILP) Mineralization Process to Normal and Pathological Biomineralization

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4.1 INTRODUCTION

The biological process by which living organisms synthesize the complex inorganic materials of their hard tissues is commonly referred to as biomineralization. Using the terminology first introduced by Lowenstam [1], biomineralization can be categorized as either a biologically induced or a biologically controlled crystallization process. Either can be associated with bioorganic materials (e.g. cell membranes, proteins and polysaccharides), but the primary distinction between these two processes is based upon the degree of specificity and control exerted during the interaction between the mineral and organic constituents throughout the precipitation process. Biologically induced precipitates occur when regions within or around the organism become supersaturated with respect to mineral salts, and often the organic component, such as, for example, the outer cell membrane of bacteria [2–5] provides a favorable surface that initiates heterogeneous nucleation of the mineral crystals. The resultant crystals may be randomly organized, with shapes that are not usually modified significantly from the inorganic habit of the mineral phase (i.e. synthetic crystals grown in the absence of organic additives), and can often be recognized by the facets common to that crystal structure. Of medical relevance is when this biological environment is the physiological media within the human body, because if this becomes supersaturated with mineral salts, pathological biomineralization can

occur, such as in atherosclerotic plaque, biomaterial encrustation (e.g. heart valve replacements, catheters) or dental and urinary calculi (e.g. kidney stones).

On the other hand, crystals produced during a biologically controlled mineralization rarely exhibit the inorganic crystal habit, and in fact, exhibit nonequilibrium morphologies (i.e. shapes) which can not really be identified as a crystal habit at all (Figure 4.1). In other words, well defined crystallographic planes

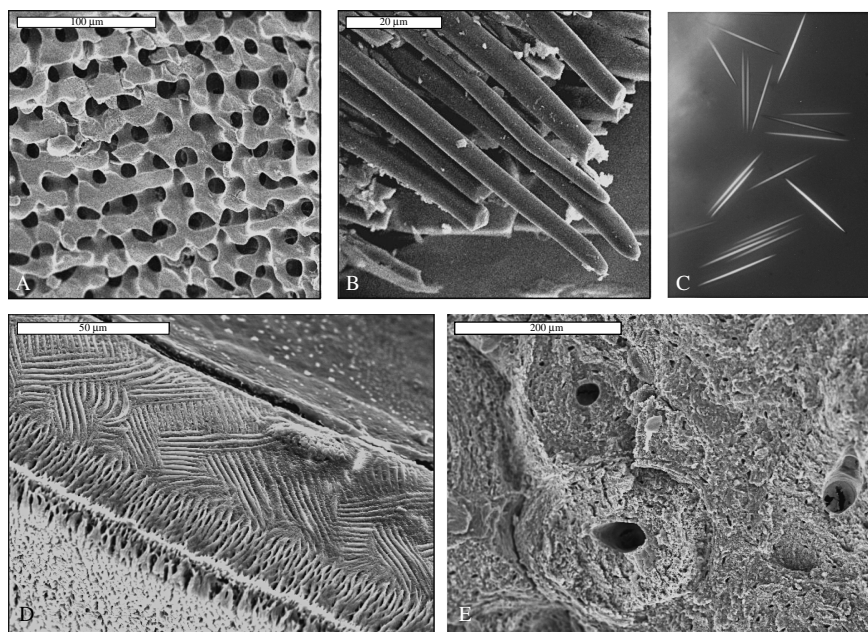


Figure 4.1 Examples of calcium based biominerals found in invertebrates, vertebrates and plants. (A) The spine of a sea urchin, which has a convoluted bicontinuous structure, is reportedly composed of single-crystalline Mg-bearing calcite. Other invertebrates, such as coccolithophorids and foraminiferans, also have ‘molded’ single-crystalline calcite morphologies. (B) The tooth of a sea urchin is composed of rods of calcite embedded within an amorphous calcium carbonate matrix. (Copyright 2003 from A New Paradigm for Biomineral Formation: Mineralization via an Amorphous Liquid-Phase Precursor, *Connective Tissue Research* 44 (Suppl. 1) 326–334 (2003) by Olszta MJ, Odom DJ, Douglas EP, Gower LB. Reproduced by permission of Taylor & Francis Group, LLC., <http://www.taylorandfrancis.com>.) (C) The needle-like crystals of calcium oxalate monohydrate seen in this polarized light micrograph are called raphides. The raphides grow in bundles within vesicular compartments within the leaves of plants. Other CaOx morphologies can be found in plants and fungi, such as acicular needles, bipyramids, large styloids or clusters in druses (similar to the cluster in Figure 4.3D). (D) Vertebrates also have fibrous minerals in the enamel portion of their teeth, although the ‘rods’ are composed of polycrystalline hydroxyapatite. In rat enamel, the ‘rods’ are woven into a triple-ply architecture, as seen in the upper portion of this micrograph. (E) The skeleton of vertebrates is also composed of hydroxyapatite, although the crystals in bone are very small and embedded within the collagen fibrils, which are arranged in concentric lamellae around the vasculature into structures called osteons (the holes are remnants of where the blood vessels traversed).

that normally arise from the lowering of surface energy are not expressed. Instead, very elaborate and complex crystal morphologies are produced, often being 'molded' within the vesicular compartment from which they are formed. This can be seen for a variety of minerals that are biologically produced, with prominent examples including the calcium carbonate spicules [6–8] and spines of sea urchins [7,9–12], and the calcium oxalate raphides in plants [13,14]. This biomineral 'molding' capability is not just limited to calcium based biominerals, but can also be seen in the ornate strontium sulfate biomineral of *acantharia* [15], the iron oxides of magnetotactic bacteria [16,17], and the siliceous spicules of marine sponges [18,19], to name a few. In most of these cases, the crystals have curved surfaces, which is highly energetically unfavorable. The fact that such morphologies are species specific also indicates that the mineralization process is under genetic control, where the proteinaceous additives produced by the cell are apparently designed to modulate various aspects of the mineralization process. Our goal, as well as many others in the biomimetics field, is to understand how cells achieve this remarkable ability, and in particular, how proteins are used to modulate the growth of inorganic crystals.

The means by which an organism can regulate the properties of its hard tissues are fairly limited (from a materials engineering perspective), because the temperature and pressure, and even pH and reaction species, are limited to fairly specific values when operating under physiological conditions. Basically the only stiffening and strengthening materials available to biological systems are the minerals precipitated from ionic salts, the majority of which are calcium based salts, such as calcium carbonate in invertebrates, calcium phosphate in vertebrates and calcium oxalate in plants. These minerals are normally quite brittle by themselves, but when combined with organic materials to form nanostructured composites, Mother Nature has found a skillful means for optimizing the mechanical properties of these biocomposites. The organic constituents are not only important for providing ductility and toughness to the hard tissue, but they are also used to regulate the formation of its biomineral constituents. Our view of the general biomineralization process is shown schematically of Figure 4.2. In order to achieve certain biomineral properties, the precipitation process is regulated by cellular processes both in time and in space, usually through selective addition of the reactants and additives to the crystallization medium. Compartmentalization is often used to accomplish this spatial and temporal control of the reactants, such as precipitation of the biomineral within membrane bound vesicles, or in the case of extracellular calcification, within a proteinaceous matrix (such as collagen in bone).

The formation of biominerals, such as bones, teeth and seashells, are not only studied because of their medical relevance, but they have also been examined with respect to their materials properties because these biocomposites (consisting of both inorganic and organic constituents) provide possible strategies for the formation of mechanically superior ceramic composites [20–26]. For this reason, many review articles on biominerals have been written from this biologically controlled perspective. However, not all biomineral composites are useful, and the study of pathological biocomposites is equally important towards understanding bone and

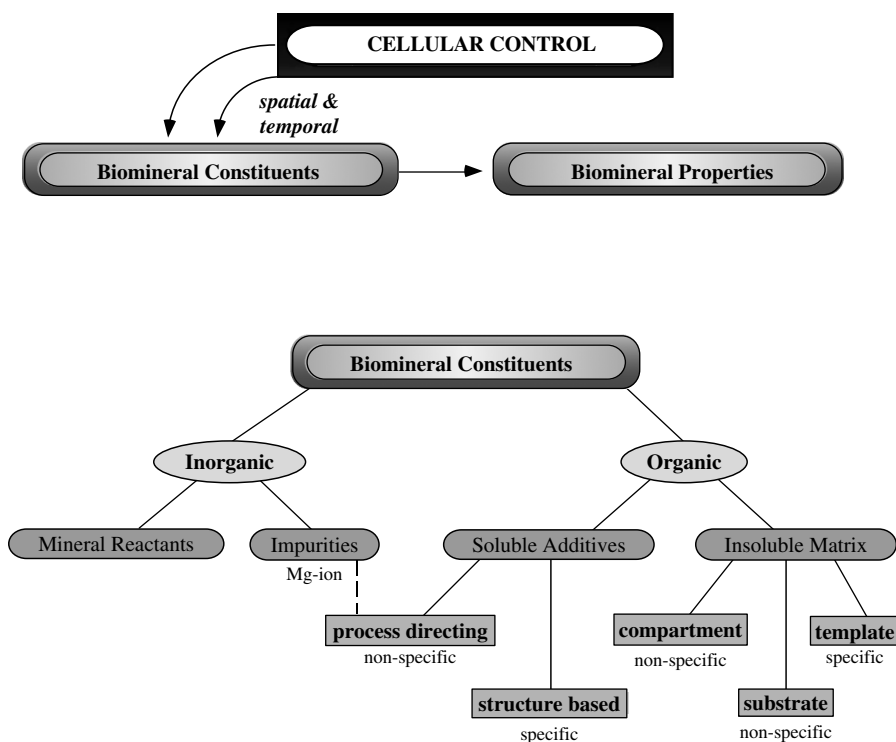


Figure 4.2 Organisms regulate the properties of biominerals, such as crystal size, shape, orientation, texture (single vs polycrystalline) and location, through spatial and temporal control over the biomineral constituents throughout the crystallization process. The constituents are usually categorized according to the purported function they play in the biomineralization process. The focus of this review is on the process-directing role of the soluble acidic macromolecules (glycoproteins and polysaccharides) found to be intimately associated with most biominerals.

renal disease states, with the hopes of eventually eradicating such pathologies. We hope to present a somewhat different perspective on biomineralization, with emphasis on the role of amorphous precursor phases, which may be relevant to both biologically controlled mineralization (in bones and teeth) and biologically induced mineralization (in kidney stones).

It should be pointed out that the supersaturation of the biological media is often not far removed from that of equilibrium conditions. This is evidenced by *in vitro* crystal growth assays using simulated body fluid (SBF) conditions, which yield faceted crystal morphologies typical of the equilibrium inorganic habit (Figure 4.3). Likewise, the crystals usually nucleate heterogeneously on any favorable substrate present in the crystallizing solution, indicating that the supersaturation is not high enough to surmount the energy barrier required for homogeneous nucleation (e.g. the hill on pathway (A) of Figure 4.4 might be smaller in the presence of a nucleating

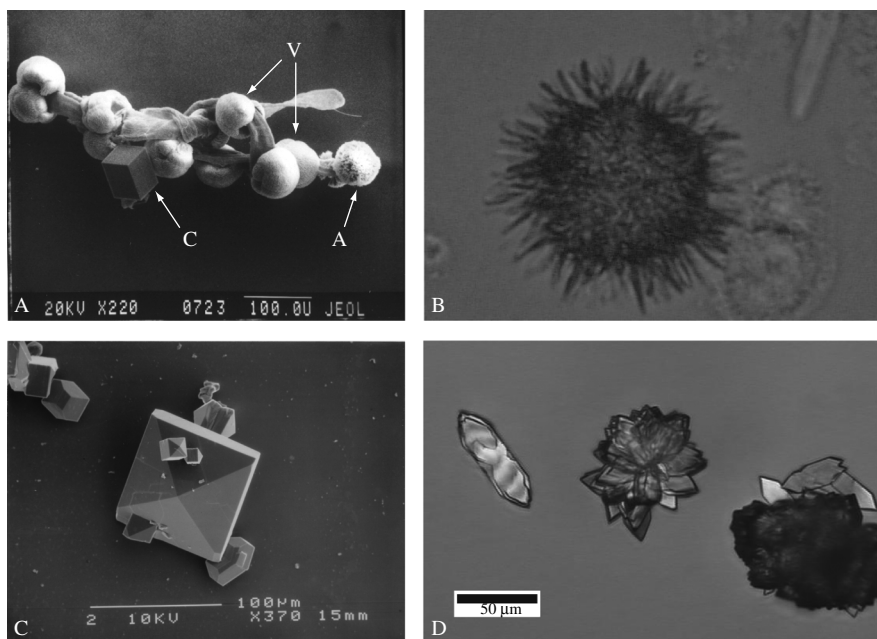


Figure 4.3 (Plate 1) The inorganic crystal habits of the three calcium-based minerals that are commonly found in biological hard tissues. (A) This SEM micrograph shows all three anhydrous phases of calcium carbonate which nucleated on a piece of dust in the crystallizing solution. Calcite is seen as the well faceted rhombohedron (indicated by C), an aragonite spherulite composed of orthorhombic needles is marked with an A and the donut-shaped spherulites are vaterite, marked with a V. (B) Calcium phosphate commonly forms spherulitic clusters of hydroxyapatite needles. This spherulite was originally an amorphous globule (like the one to the bottom right), which then recrystallized into needles of HA. (C) Calcium oxalate most commonly occurs as tetragonal bipyramids of calcium oxalate dihydrate (COD), or (D) as coffin-shaped monohydrate (COM) crystals which are often twinned. Excessive twinning leads to clusters and spherulites, as seen here.

surface). This is useful, because if the organism were to operate under conditions of high supersaturation, crystals would nucleate homogeneously in the solution, making it difficult to regulate their location and properties. At moderate levels of supersaturation, such as in the metastable regime, the thermodynamic driving force is not sufficient to overcome the energy barrier for homogeneous nucleation. Therefore, crystal nucleation can be directed by a substrate which provides a lower energy barrier for heterogeneous nucleation, which in a biological system may consist of a phospholipid membrane, or a proteinaceous or polysaccharide matrix.

Although this review is focused on the role of organics (mainly biomacromolecules) in biomineralization, it should be kept in mind that inorganic species aside from the primary mineral phase, which will be referred to as impurities or additives (depending on whether the mineralization is induced or controlled), can also play a decisive role in the crystallization process. Magnesium

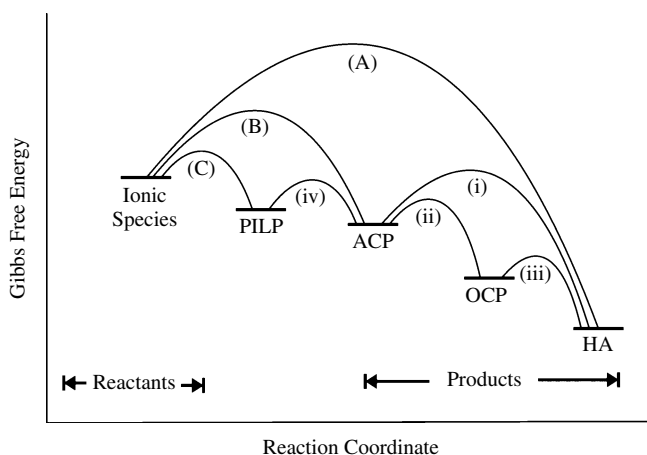


Figure 4.4 Different mechanisms for lowering the Gibbs free energy of the system can give rise to different crystallization pathways. Calcium phosphate (CaP) is used as an example here, but it should be noted that the energy levels of the different CaP phases and the heights of the energy barriers are not to scale. Pathway (A) represents the traditional nucleation of crystals, such as *de novo* precipitation of HA directly from the calcium and phosphate ionic species present in the parent solution. Pathway (B) represents crystallization via an amorphous precursor phase, which might occur if the energy barrier for nucleation is too high to follow pathway (A). An amorphous precursor (ACP) is common for calcium phosphates, which then transforms into either HA or OCP, via pathway (i) or (ii) respectively. OCP may occur as a transient precursor to the more thermodynamically stable phase of HA (iii), or if the energy barrier is large, it may persist, particularly if the sample is dried and does not allow for recrystallization. Pathway (C) differs from (B) in that the polymeric additive stabilizes an amorphous PILP phase with fluidic character, which then solidifies into ACP (iv), and ultimately transforms into one of the more stable crystalline phases, such as HA (i).

ion is a notable example because it is found at quite high concentrations in seawater [27] and most physiological solutions, so its presence should not be ignored. In fact, magnesium is substituted into some calcitic biominerals at quite high levels (e.g. Mg-bearing calcite is found in echinoderms and calcitic sponges) [28], as well as in the phosphatic biomineral of bones and kidney stones (as whitlockite, β -(Ca,Mg) $_3$ (PO $_4$) $_2$). Unfortunately, studies with both organic and inorganic additives are limited [28–30] because this adds another level of complexity to understanding the biomineralization process. In our calcium carbonate experiments, in which we have added magnesium ion additive, a strong synergistic effect is observed with the combination of acidic polypeptide additive in the presence of magnesium ion to form amorphous precursor phases, as will be discussed shortly.

4.1.1 CRYSTALLIZATION MECHANISMS

When considering biomineralization, or any crystallization process for that matter, it is often convenient to think of it in terms of the stages of crystal nucleation

and growth (and aggregation in some cases). Indeed, most review articles present biomineralization in this fashion, according to classical crystal growth theory. A notable exception to this can be found in the papers by Mann [15,31,32], who describes the different pathways from which a crystallization reaction can proceed. Figure 4.4 is a modified version of the schematic that Steve Mann has used to illustrate crystallization via a precursor pathway. Our schematic contains an additional new precursor phase, which will be discussed shortly. The way the precursor pathway works is that, if a solution is supersaturated with respect to multiple crystal phases, then often times the first phase to precipitate out is the most soluble (least stable) phase. Given that the first formed precipitate is not the most thermodynamically stable phase, it will then transform or recrystallize into the more stable phase to lower the free energy of the system (Figure 4.4, Pathway B). There could be several phases of intermediate stability before finally arriving at the crystal that provides the lowest free energy (e.g. Figure 4, Pathway B (ii) then (iii)), but often times the pathway just consists of one precursor that transforms directly to the most stable state (Figure 4, Pathway B (i)). The path that is followed will depend on the relative heights of the energy barriers to reach each state, balanced by the overall energy available to the system (i.e. the strength of the thermodynamic driving force). Such precursor pathways are well documented for many mineral systems [33–38], and these empirical observations, which arise due to kinetic effects, are often referred to as following the Ostwald–Lussac Rule of Stages [39].

This review will present evidence (from *in vitro* model systems) to suggest that an important aspect of biomineralization may be crystallization via metastable amorphous precursor phases. At first thought, one might not expect amorphous phases to occur under conditions that are not far removed from equilibrium, which is generally the case in physiological solutions, as mentioned earlier. Amorphous phases are normally formed at very high supersaturation (and/or at low temperature), when the reaction becomes kinetically dominated such that there is not sufficient time for organization of the ions into a crystalline lattice. An alternative way to frustrate the organizational activity is to include impurities, and both organic and inorganic impurities can inhibit the crystallization process to induce amorphous phases [28,40,41], even under relatively mild supersaturation conditions. It may be more appropriate to say that impurities or additives stabilize, rather than induce the amorphous phase, in light of recent evidence from *in situ* synchrotron X-ray small- and wide-angle scattering experiments, which indicate that the first formed nucleus in the precipitation of calcite is amorphous, even without additives [42,43]. Nevertheless, given the very short-lived occurrence of this unstable amorphous embryo in these experiments, the important point is that induction or stabilization of the amorphous phase by an additive leads to lifetimes that are readily observable by standard techniques. Even more importantly in terms of application, lifetimes of the metastable phase can be long enough to allow for manipulation of the precursor phase (such as molding or shaping within vesicles).

When a crystallization reaction proceeds through an amorphous precursor, this can have a pronounced effect on the resulting crystal morphologies [44];

therefore, crystal growth *via* an amorphous precursor phase should be considered as a distinctly different mechanism than the traditional solution nucleation and growth mechanism, and especially when considering the crystallization processes involved in biomineralization, for which organic and inorganic additives/impurities are bountiful. Note, the terminology here becomes somewhat tricky, because any phase, including an amorphous phase, can be ‘nucleated’. In addition, crystals can nucleate and grow from within the amorphous phase; in this case, we will refer to this as a phase transformation, to avoid confusion with the more common usage of the word ‘nucleation’ in a traditional crystallization process.

The presence of amorphous phases in biominerals, either as transient precursors or stabilized structures, has been receiving more and more attention in recent years [33,35,36,41,45–49]. Although most of the recent literature on amorphous phases has focused on calcium carbonate (CaCO_3) biominerals which are prevalent in marine invertebrates; of greater significance to vertebrates are the biominerals of calcium phosphate (CaP) and calcium oxalate (CaOx). These two minerals are the primary constituents of bones, teeth and kidney stones (the latter of which contains both CaP and CaOx). While bones and teeth are clearly examples of biologically controlled mineralization, kidney stones are the unfortunate opposite scenario of being biologically induced minerals. The concept of an amorphous precursor in the calcium phosphate biominerals is not new, and was first proposed by Eanes and Posner [50–52]. Around the same time, Bonnucci [53,54] also presented strong *ex vivo* evidence to support such claims, in which an ‘inorganic substance within bands’ can be clearly seen in his TEM images of unstained collagen fibrils of the early stages of bone formations. Notwithstanding, this has remained a hotly debated issue, so here we present results from our *in vitro* model systems, using CaP and CaOx, to support the hypothesis that amorphous precursors play a key role in these medically important biominerals. Even though CaCO_3 biominerals are only found in the inner ear of humans, we will also include some examples from our studies on the CaCO_3 model system because we have accumulated extensive data with this system [36,44,48,55–65]. The relevance of such *in vitro* studies lies in understanding the mechanism of crystallization via an amorphous precursor, and such concepts extend beyond specific mineral compositions.

4.1.2 *IN VITRO* MODELS OF BIOMINERALIZATION

It is extremely difficult to examine biomineralization reactions directly. This is most certainly the case for vertebrates, which do not provide the opportunity to study biomineral formation *in situ*, as has been done for the CaCO_3 biominerals of small marine organisms, such as the spicules in sea urchin larvae, in which spiculogenesis can be directly observed on an optical microscope [6,66–68]. Notably, this is one of the first examples for which it was discovered that the biological calcification proceeds via an amorphous CaCO_3 precursor (along with sponge spicules and mollusk larva, and most recently adult sea urchin spine regeneration) [40,69–71].

For most organisms, researchers do not have the ability to directly examine biomineralization processes *in vivo*. Therefore, it should be cautioned that most of the theories on biomineralization (including the ones presented here) are based on analyses that use *in vitro* crystallization assays. Even though they are limited in scope, *in vitro* assays provide the advantage of allowing one to examine the influence of various additives on the crystallization process, and clarify the role of species isolated from a biological system. Such analyses, and particularly observations of modified crystal morphology, may then be correlated to features seen in biogenic minerals. One must always keep in mind that *in vitro* assays use a much simplified environment compared to the physiological system, which is dynamically changing as the cells modulate the composition of the crystallization medium both in time (from ion channels across membranes) and in space (via compartmentalization of reactions within membrane-bound vesicles or macromolecular matrices) [72–74]. In addition, isolation of the variables also eliminates the possibility of cross interactions, which as previously mentioned for the case of Mg ion and polymer, can be significant. Studies using experimental design techniques can be useful in this regard when dealing with complex multivariable environments, such as the urinary tract, for example [75,76]. Regardless of the shortfalls of *in vitro* models, valuable information can still be obtained, and this is the approach we have adopted.

In some biomineralization studies, protein constituents have been extracted from various biominerals, and then added to *in vitro* crystallization assays [19,77–82]. This is done in order to examine their influence on either the nucleation or growth of the same mineral, but without the complicated assortment of species present in the physiological solution. These types of studies using protein extracts, or simpler mimics to such biopolymers (e.g. polypeptides) [44,83–85], have shown that biopolymers can have a pronounced effect on crystal nucleation and growth. The macromolecular constituents of biominerals are often described as being one of two types, insoluble matrix macromolecules and soluble acidic macromolecules (Figure 4.2). The latter is more the focus of this review, although the insoluble matrix is clearly important for providing the template and/or compartment which regulates where the inorganic phase is formed, as well as influences the crystallographic phase and orientation [15,32].

Less clear is the role of the soluble acidic macromolecules that are found to be intimately associated with (and even occluded within) biominerals. These soluble polyanionic proteins are found to be ubiquitous in biologically controlled mineralizations [10,78,86,87], and are present in pathological biominerals as well [88]. Therefore, many researchers (including ourselves) have examined the influence of the acidic polymers on crystal growth. The acidic proteins seem to behave differently in solution than most proteins, with some studies indicating that they do not possess much second or higher order structure (such as β -sheet or α -helix), as one might find in a globular protein [89,90]. The reason for this may be that they are well solubilized since they contain so many acidic residues, more so than any other proteins found in the body, being highly enriched with acidic residues such as aspartic and glutamic acid, and phosphoserine [10,78,87,91,92]. Glycoproteins and

polysaccharides can also contain many acidic residues (including sulfate-containing saccharides), and even though they are also found associated with biominerals, most of the biomineral research community has ignored them due to their complexity. This is understandable given the difficulty of synthesizing, or even extracting and characterizing such macromolecules; but our concern is that these species could be involved in the crystallization process, yet the presence of glycosylated side groups is usually neglected when researchers try to model molecular level interactions of inorganics with protein domains. That said, we will ignore them as well since our lab is not equipped to deal with such complex macromolecules; but because we are not modeling molecular recognition at the organic–inorganic interface, this should be acceptable. One might anticipate that substantial interactions could occur between the charged carboxylate, phosphate and sulfate groups of such biomacromolecules with ionic crystals, and indeed much biomineralization research has focused on this interface. Our focus has been on the early stage macromolecular interactions with ionic precursors (prior to the formation of a crystal interface), which we find can provide a powerful means of modulating crystal growth through the formation of amorphous precursors. We refer to this type of additive as a process directing agent (Figure 4.2).

4.1.3 INFLUENCE OF ADDITIVES ON CRYSTAL NUCLEATION

In studies on the influence of additives on crystal nucleation, the terminology of promoter or inhibitor is often used to describe the effect of the additive, which is frequently measured by the induction time to nucleation. Such studies are directed at learning whether the role of the additive is to stimulate nucleation at a certain time or location, with the idea that this type of regulation would be of value towards building hierarchical tissue structures, such as in the case of the biologically controlled mineralization of bone. On the other hand, in the case of biologically induced mineralization, such as urolithiasis, the additive that stimulates crystal nucleation or growth may be likely to promote stone formation, while an inhibitor might be specifically secreted by renal epithelial cells to block crystal nucleation, growth or aggregation. Such assertions seem reasonable, but unfortunately, this bipolar view of inhibitor vs promoter leaves off the intermediate scenario, where we have found that the inhibitory action of acidic polymers can lead to a *delay* of nucleation, and when the nucleation event finally does commence, it is not for a crystal, but rather an amorphous phase [44]. Therefore, the terminology inhibitor vs promoter does not adequately describe the role of the polymer, which transforms the crystallization process into a precursor process. For this reason, even though some acidic polymers may act as inhibitors, we prefer to call them process directing agents.

Polymer-Induced Liquid-Precursor (PILP) Process

It is well documented that acidic proteins and polymers are often inhibitors of crystal nucleation [93–98], including polyaspartic acid [77,85], a commonly studied

biomimetic polymer. We have also examined the influence of polyaspartate on mineralization, and our initial observations matched those described in the literature [77], in which the polymer only led to nonspecific interactions, producing aggregates of rounded crystals (Figure 4.5A), when added at low levels to a crystallizing solution of CaCO_3 . We then came to realize that under certain solution conditions,

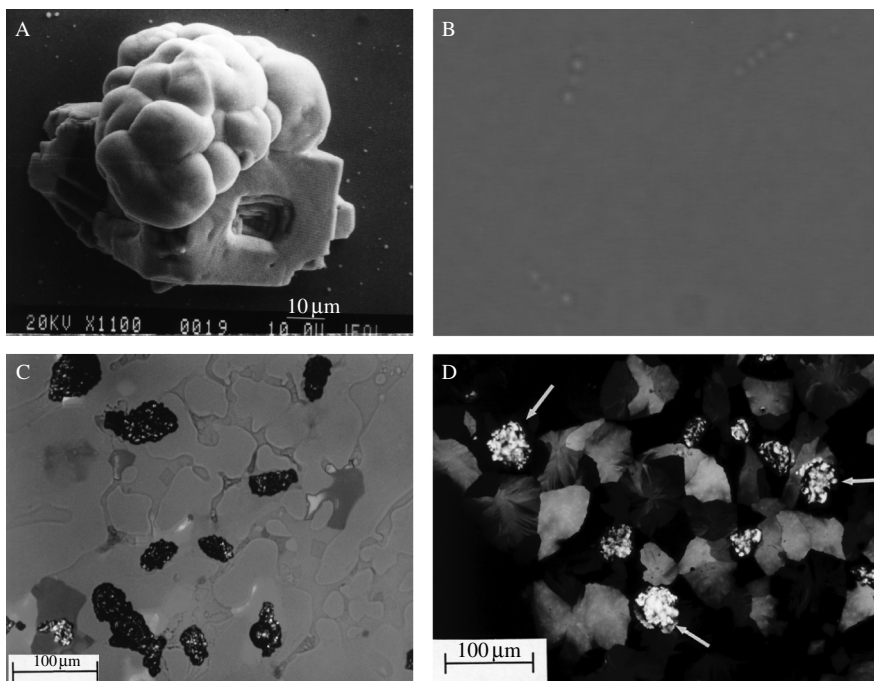


Figure 4.5 (Plate 2) Calcium carbonate morphologies produced during the PILP process. (A) With low levels of polyaspartate additive, aggregates of rounded crystals are produced. The round crystals have been determined to be spherulites of vaterite, which are smooth because they are coated with a ‘membrane’ of mineral film. Even the faceted rhombohedral crystals of calcite appear distorted, and are considered hybrid crystals, which are presumably formed from the traditional solution growth, but become distorted by the presence of the fluidic amorphous precursor. (B) Droplets of the PILP phase can be seen in the early stage by *in situ* examination of the reaction when the solution becomes cloudy. Aggregation of the droplets is often observed, and here the droplets ($\approx 2\mu\text{m}$ in diameter) appear to be congregating into linear chains. (C) The precursor phase has deposited as a swirly film on the glass coverslip, and is beginning to transform. The amorphous films appear the same magenta color as isotropic background, while the orange and blue (go gators!) patches are single-crystalline calcite. Many aggregates are also present, and appear dark since they are much thicker than the thin films. (D) After transformation, the films appear fully birefringent, except the patches that are oriented in the extinct position. The arrows point to aggregates of calcite, which are nearly always present unless the reaction is performed at low temperature (4°C), or in the presence of Mg ion additive. (Reprinted from *Journal of Crystal Growth*, Vol. 191, Gower LA, Tirrell DA, Calcium Carbonate Films and Helices Grown in Solutions of Poly(aspartate), 153–160, Copyright 1998, with permission from Elsevier.)

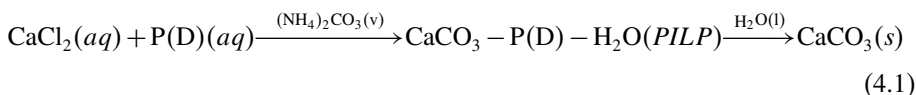
the inhibitory action of the charged polymer (of relatively low molecular weight, in the range of 5–15 000 Da) can transform the reaction into a precursor process [44]. In other words, the charged polymer sequesters a high concentration of ions; yet inhibits nucleation within this localized, highly supersaturated environment, such that a critical point is reached to induce liquid–liquid phase separation. Clouding occurs within the solution as droplets of the precursor phase are formed during the L–L phase transition (Figure 4.5B), and this is only seen to occur upon the addition of the counterion (e.g. carbonate or phosphate). In other words, this is not a ‘salting out’ effect, which commonly occurs for many proteins in solutions of high ionic strength, but instead a new phase is formed which retains a significant fraction of hydration waters, imparting the amorphous precursor with a fluidic consistency.

It should be noted that the formation of metastable phases was documented long ago in the Ostwald–Lussac rule of stages [39], but mention of a liquid phase seems to be lost in translation in most recent references to this empirical rule. In the classic book *On Growth and Form* [99], D’Arcy Thompson discusses the formation of rounded concretions, and states the following:

In accordance with a rule first recognized by Ostwald, when a substance begins to separate from a solution, so making its first appearance as a new phase, it always makes its appearance first as a liquid.

Droplets of this *polymer-induced liquid-precursor (PILP)* phase are initially nanoscopic, and only detectable by light scattering, but they grow in time and accumulate to become a few microns in size, at which point they can be seen on an optical microscope (Figure 4.5B). The size plateau we observe *in vitro* is probably limited by the polymer and reactant ion concentration in the solution, which may differ for different mineral systems with differing solubility products. In addition, the droplets solidify with time, which imparts a size limit depending on how long they stay fluidic enough to aggregate and coalesce into larger sizes. In the limited spatial compartments of biominerals, one would not expect to see such large droplets, but this *in vitro* system makes the study of this unusual precursor process accessible. One can first examine the droplets *in situ* on an optical microscope when clouding occurs; and then when the droplets settle and coalesce into films, one can examine the transformation of the amorphous phase (Figure 4.5C). The films are initially clear and nonbirefringent when examined by polarized light microscopy (isotropic amorphous materials are not doubly refracting), and then the films become birefringent as they crystallize into the more thermodynamically stable crystalline phase (typically calcite for CaCO_3 , or hydroxyapatite for CaP), as shown in Figure 4.5D.

The stages observed for the polymer-induced liquid-precursor (PILP) process are schematically illustrated in Figure 4.6 [44], and can be summarized for the CaCO_3 system as follows:



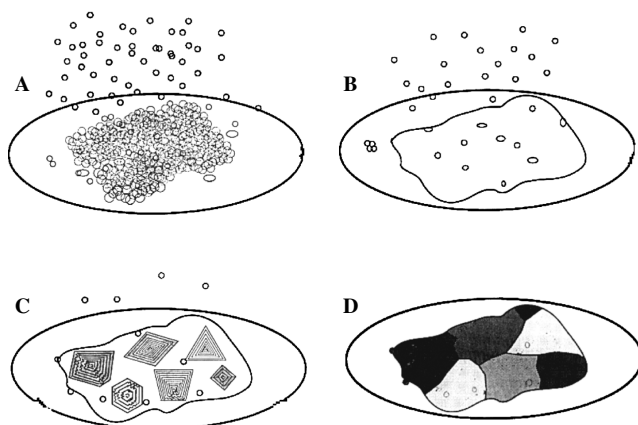


Figure 4.6 Illustration depicting the stages and features of the PILP process. (A) As a critical concentration is reached during the infusion of the carbonate species, isotropic droplets ($\sim 2\text{--}5\ \mu\text{m}$ in diameter) phase-separate from the solution and accumulate on the glass coverslip. (B) The droplets coalesce to form a continuous isotropic film. Some late-forming droplets may be partially solidified, or crystalline, and do not fully merge with the film. (C) Patches within the isotropic film become birefringent as crystal tablets nucleate and spread across the precursor film. The transformation sometimes progresses in an incremental fashion, delineating sectors within the calcite tablets. The incremental steps arise from exclusion of the polymeric impurity, and lead to transition bars, which become birefringent more slowly due to the impurity. (D) The tablets continue to transform as the crystals grow laterally to form a continuous film. The transformed film is about half a micron thick, and composed of single-crystalline patches of calcite, which range from tens to hundreds of microns in diameter. (Reprinted from *Journal of Crystal Growth*, Vol. 210, Gower LB, Odom DJ, Deposition of Calcium Carbonate Films by a Polymer-induced Liquid-Precursor (PILP) Process, 719–734, Copyright 2000, with permission from Elsevier.)

We first discovered the PILP process for CaCO_3 using the acidic polypeptide, polyaspartic acid (sodium salt), which is considered a simple mimic for the acidic proteins extracted from biominerals. We have since come to realize that polyacrylic acid, or other acidic macromolecules, will produce similar effects, and that other minerals can also be influenced similarly by acidic macromolecules.

The fluidic nature of the amorphous precursor phase can have interesting consequences with respect to crystal morphology and aggregation, which we believe may be relevant to biominerals, both in invertebrates and vertebrates. For example, we have been able to reproduce (quite often, accidentally) many of the features found in biominerals using the PILP process, including thin CaCO_3 tablets [55] with defect textures similar to seminae of bryozoans [100], fibrous calcite (Figures 4.7A and B) [48,101], molded single-crystalline calcite (Figures 4.7C and D) [58,62–64], and the nanostructured architecture of bone [48,102–104] (which will be described further).

Amorphous Phases

There is a variety of ways to produce amorphous phases, such as by just pushing the kinetics with ultra-high supersaturation. Due to the limited solubility of calcium

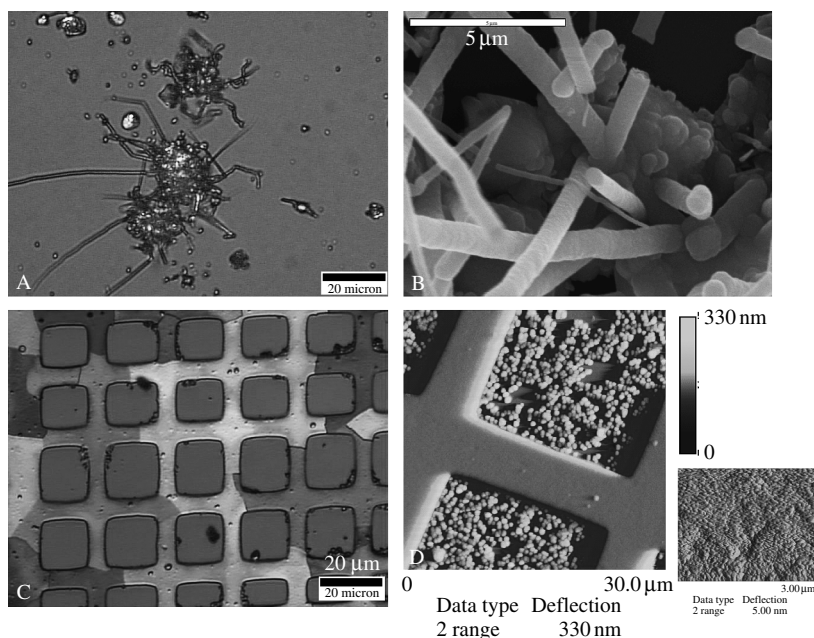


Figure 4.7 (Plate 3) Examples of nonequilibrium crystal morphologies produced for calcite crystals formed by the PILP process. (A) Fibers of calcite were ‘extruded’ from an amorphous globule of PILP phase. The uniform retardation color indicates they are single-crystalline (as well as electron diffraction spot patterns of isolated fibers). (B) Scanning electron microscopy shows the structure of the fibers, which clearly are not needles (as might be found for aragonite). Note, the small fiber in the middle is ‘draped’ across the larger fiber, suggesting that the shape was formed before the precursor material fully solidified. (For Figures 4.7A and B, Copyright 2003 from *A New Paradigm for Biom mineral Formation: Mineralization via an Amorphous Liquid-Phase Precursor*, *Connective Tissue Research* 44 (Suppl. 1): 326–334 (2003) by Olszta MJ, Odom DJ, Douglas EP, Gower LB. Reproduced by permission of Taylor & Francis Group, LLC., <http://www.taylorandfrancis.com>.) (C) The soft lithography technique of microcontact printing was used to pattern a gold-coated substrate with self-assembled monolayers (SAMs) of alkane thiols. The PILP phase preferentially deposited on the carboxylate terminated SAMs, yielding a patterned film of calcite. In essence, single-crystalline patches of calcite were ‘molded’ by spatial delineation of the templating substrate. (Reproduced by permission of Kim Y, Gower LB, *Formation of Complex Non-Equilibrium Morphologies of Calcite via Biomimetic Processing*, *Materials Research Society Symposium Proceedings* 774: 141–148 (2003).) (D) Atomic force microscopy deflection images demonstrate that the patterned calcite films were generated from colloidal droplets of the precursor phase. Large droplets sometimes deposit in the nonpreferred region, as can be seen in the middle of the grid pattern. These droplets were more solidified than the fine-scale colloids that deposited earlier in the preferred region (inset at the bottom right was taken from the smooth grid region of calcite).

phosphate, the formation of an amorphous gel is a common occurrence, while in the case of calcium carbonate, the amorphous phase is very unstable and seldom seen. As mentioned above, physiological solutions are usually not highly supersaturated, and this is likely by design, because careful control of ion balance must be

maintained for physiological function, as well as for avoidance of undesirable precipitates. Therefore, biological systems apparently use additives to provide the high level of control that is needed for precipitating amorphous phases when and where they are needed. The additives most commonly used to produce amorphous calcium carbonate (ACC) appear to be acidic polymers, along with magnesium or phosphorous-containing ions [105]. Interestingly, a correlation was noted for the acidic polymers extracted from biominerals, in which those proteins associated with the more stable biogenic ACC (plant cystoliths and crustacean gastroliths) are enriched in glutamic acid and/or glutamine, while the proteins associated with the transient ACC precursors (sponge and ascidian, and probably many others) seem to be enriched with aspartic acid and/or asparagine.

It has yet to be determined whether any amorphous phases in biominerals are fluidic; but the fact that such a phase can be created *in vitro*, and with relative ease (simply through the addition of 'nonspecific' acidic macromolecules), and the fact that several crystallographic features of biominerals can be duplicated, suggest that the PILP process may play a key role in biomineralization. The ability to physically distinguish between an amorphous solid vs amorphous liquid phase is not something that can be easily demonstrated given that both are structureless materials which may only vary in the amount of water present in the phase. In addition, considering amorphous phases as structureless is not entirely accurate, as can be demonstrated by extended X-ray absorption fine structure analysis (EXAFS), which shows that differences in short-range order in biogenic ACC may correlate to the resulting crystal phase upon transformation [49,105]. Nevertheless, for discussion purposes, we will not worry about semantics, and consider a nonbirefringent mineral phase (of a doubly refracting mineral), which does not yield X-ray or electron diffraction peaks, to be an amorphous precursor. Even though it may have short-range order (such as average interatomic distances), the primary point of interest here is that this amorphous (or paracrystalline) phase serves as a precursor to the final crystalline product, and it is the crystallization pathway that is significant in determining properties of the final crystals. The differences in short-range order of biogenic ACC, as well as our own *in vitro* observations of ACC (Figure 4.8), indicate that ACC is not structurally one mineral phase. Therefore we will discuss the potential consequences of this newly identified amorphous PILP phase, and the biological cases where the fluidity of the precursor phase is thought to be significant will be highlighted.

4.1.4 INFLUENCE OF ADDITIVES ON CRYSTAL GROWTH

In the case of crystal growth, it is well recognized that additives can influence crystal growth to produce a change in crystal morphology. Proteins extracted from biominerals have also shown a pronounced influence on crystal growth, and it has been proposed to occur through selective binding of the additive to specific crystallographic planes [77–79]. The ability to control crystal shapes with selective binding is particularly well documented with small molecule additives [106], and

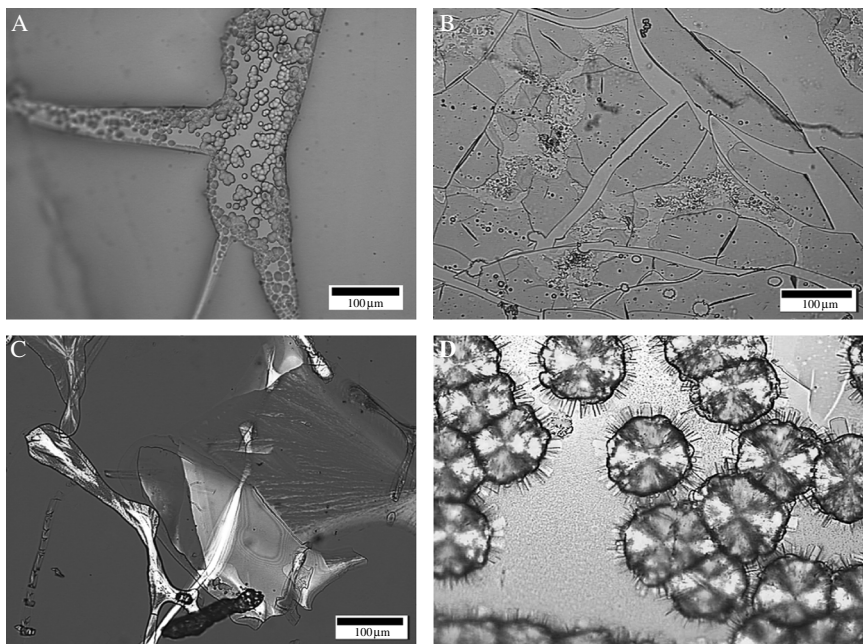


Figure 4.8 (Plate 4) Structures and transformation of amorphous calcium carbonate (ACC). (A) Coalesced droplets of PILP phase can be seen at the edge of this spongy looking film, which apparently contains much more water than the glassy ACC film shown in (B). Both of these films were formed at the air–water interface under Langmuir monolayers of steric acid. The film in (B) cracked as it was dipped off the air–water interface onto a glass slide, showing the brittle nature of its glassy composition. (C) This ACC film is beginning to transform by the pseudo-solid-state crystallization mechanism. The solid orange-yellow region is transforming into single-crystalline calcite, while the striped region is transforming into spherulitic vaterite (as a two-dimensional spherulitic film). (D) This film seems to be transforming via dissolution and recrystallization into three-dimensional spherulites, although the outer edges of the spherulites appear to be crystallizing by the pseudo-solid-state transformation.

has been used in industrial processes. As an example, Figure 4.9 shows how easily the crystal habit of calcium oxalate can be modified by various additives. The same is true of calcium carbonate, where the literature is bountiful with modified crystal shapes of calcite. We have found that just about any additive will lead to a new and interesting crystal shape for CaCO_3 . But the ability to regulate crystal morphology into the complex shapes found in biominerals using this approach seems limited, given that it is not simply the expression of new faces, but rather the ‘molding’ of an entire structure, which is often not symmetrically organized (i.e. a complex assortment of additives would have to be placed in many different locations around a forming crystal).

Loste and Meldrum have nicely demonstrated that single crystals of calcite can be ‘molded’ into complex morphologies within a predefined space [37]. In their

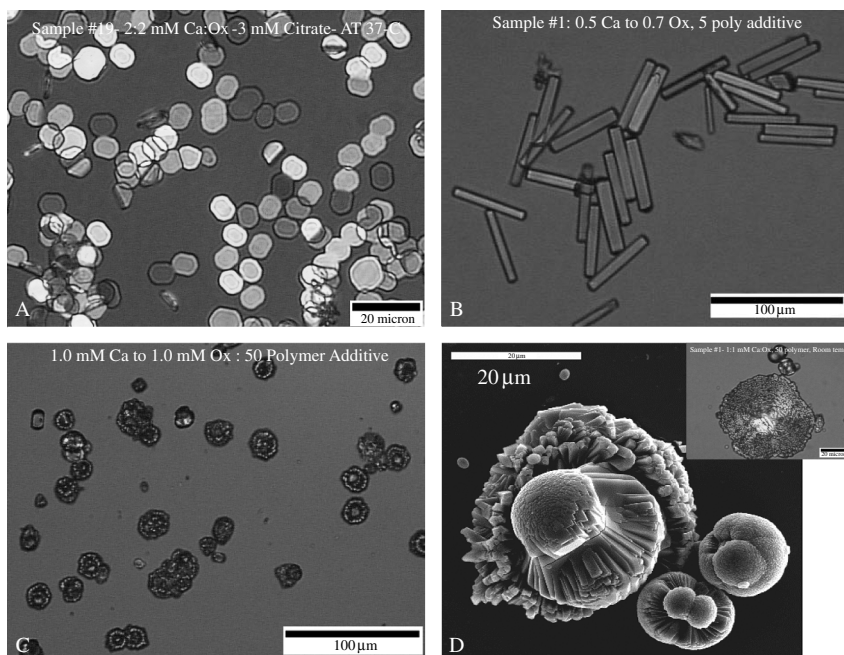


Figure 4.9 (Plate 5) Variations in calcium oxalate morphologies produced with simple crystal growth modifiers. The crystal phase has not been determined by X-ray diffraction, but they appear to all be modified COM crystals, which normally exhibits the coffin-shaped morphology (Figure 4.3D) under similar conditions, but without additive. (A) With 3 mM citrate, thin flat platelets with very rounded ends are produced. (B) Elongated prisms without triangular or rounded ends are produced with a small amount (5 $\mu\text{g}/\text{ml}$) of polyaspartic acid. (C) A higher concentration of the polymer (50 $\mu\text{g}/\text{ml}$) leads to unusual knobby aggregates, which likely recrystallized from an amorphous globule. (D) A polarized micrograph at higher magnification (inset at top right) shows the distinct Maltese cross pattern of these spherulitic aggregates, and the scanning electron micrograph shows the formation of asymmetric spherulites, which is the cause of the knob at the middle of each globule.

original work, the use of an amorphous precursor was thought to be critical, but more recently, Meldrum [107,108] has shown that it is not always necessary to mold a precursor, and that a spatially delineated calcite crystal can be generated if the nucleation event is spatially and temporally controlled. Aizenberg and coworkers [109] have also demonstrated the ability to mold single-crystalline calcite, in this case via an amorphous precursor generated by using tailored templates and inhibitory additives (Mg ion). We have also demonstrated the ability to micromold crystals from an amorphous precursor [58,63], but our work differs from Aizenberg in that we have molded a fluidic precursor phase (Figure 4.7D) [62].

Given that many biomineral deposition vesicles are dynamically changing their shape as the crystal is being formed, the concept of a fluidic precursor is appealing. In addition, it is noted that biomineral deposition vesicles usually appear to tightly

encapsulate the biomineral with membrane, leaving very little solution space surrounding the forming crystal. This makes one wonder how there can be enough ions to build the crystal in this small volume of solution? Therefore, it seems likely that this small volume contains a highly concentrated pool of ions, such as might be found in a precursor phase. Indeed, some studies indicate that the small vesicles that are being transported into the biomineral deposition vesicle contain an amorphous phase [6,68]. It seems plausible that these vesicles could be delivering a precursor phase such that it can be applied to the forming crystal in the precise location that is to be molded and built, which might be easier than directing the transport of free ions in solution. In addition, if a crystal is to be constructed by accumulation of some precursor phase delivered by these vesicles, than it seems more likely that a fluidic precursor could be slathered on, rather than granules of an amorphous solid. But this is only speculative, and one could also argue that nanoparticles of a solid amorphous precursor would be unstable and recrystallize, building upon the base crystal (such as in Ostwald ripening). This scenario, however, brings back the question of how to direct the dissolved free ions back into the proper location.

In our studies with amorphous precursor phases, we have found that if the metastable phase dissolves and recrystallizes, instead of the pseudo-solid-state transformation pathway (Figure 4.8C), it always leads to spherulitic crystals and aggregates (Figure 4.8D). Therefore, we favor the idea that the vesicular cargo is a fluidic precursor phase that is slathered on and molded into the complex morphology dictated by the shape of the surrounding membrane of the vesicle. In either case, the general concept of ‘molding’ an amorphous precursor phase, whether it be solid or liquid, seems simpler and more feasible than the selective binding mechanism, given the enormous variety of crystal morphologies expressed in biominerals, and even within the same species. This reduces the complexity down to manipulating the shape of the vesicle, and this is something that cells can do with ease. Even the simpler organisms, such as algae and protists, seem to build as elaborate structures (e.g. coccoliths) [15,110] as the more advanced invertebrates. In the evolutionary scheme of things, this reassignment of complexity makes sense.

Specificity of Interaction

The influence of a simple polypeptide, such as polyaspartate, on crystal growth has usually been considered to be ‘nonspecific’ interaction because it leads to nonspecific changes in crystal morphology, such as rounded crystals and aggregates [77]. Therefore, relatively little attention was paid to this additive until recently. The terminology ‘nonspecific’ refers to the more or less random adsorption of an additive to the high-energy edges and corners of a forming crystal, leading to rounding off of the sharp crystal facets. In contrast, a ‘specific’ interaction might occur between the additive and specific crystallographic faces due to some type of molecular recognition, where there is a match between the geometry of the inorganic crystal lattice and the functional groups on the organic additive (e.g. from the spacing or stereochemistry of the carboxylate side groups of a

protein). This type of 'specific' interaction has been the focus of biomineralization studies for many years, as mentioned above for the case of modification of crystal growth by stereoselective interactions [77–79,111]. Recently, however, atomic force microscopy (AFM) studies have demonstrated that such molecular level interactions are often occurring at step edges of the growing crystal, which also have a geometry that can favor preferential adsorption such that the growth kinetics along different crystal planes is preferentially modified [112–114]. Therefore, when considering molecular recognition between a soluble organic additive and inorganic crystal, it may be more appropriate to model crystal defects (step edges and kink sites) rather than flat crystallographic planes, which has been prevalent in the older literature.

The verdict is still out on this issue of specific molecular recognition occurring in biomineralization; but based on our studies, the point can be made that simple 'nonspecific' interactions may play a vital role in biomineralization; and that role, as suggested earlier, is as a process-directing agent. In other words, through the addition of micromolar levels of acidic polymers, the traditional solution crystallization process can be transformed into a precursor mechanism. This is a very powerful tool in terms of the ability to modulate crystal growth since the crystals retain the shape of the precursor. Consider for example that the PILP process is in many ways analogous to sol-gel precursor processes used to prepare ceramics at low temperatures. Sol-gel ceramics have been deposited as thin films and coatings, or molded into complex shapes, or extruded into fibers. This is true of the PILP process as well. The primary difference though, lies in the formation of the precursor, which is a chemical precursor in the sol-gel technique, rather than a physical precursor, as seen for the PILP process (via sequestration of ions and water by polymer).

The usefulness of nonspecific interactions is starting to be realized with respect to the ability to modulate crystal morphology via process directing agents. This does not imply that specific interactions are not involved in biomineralization; quite the contrary. The PILP process (or an amorphous phase in general) provides a relatively simple means of regulating crystal shape; but there are other features of biomineralization that are not accomplished by this process alone, such as crystallographic orientation and phase. The controlling mechanism for these aspects is not really a separate entity, but rather would occur in combination with the precursor process. For example, the possibility of regulating crystal orientation via pseudo-epitaxy on an organic template remains, but instead one might anticipate that the high ion concentration coupled with limited diffusion within the precursor phase, would lead to different nucleation behavior than crystals nucleating from solution. Indeed our preliminary studies in this area suggest this is the case (see discussion on bone).

To recap, the molecular level interactions that lead to the PILP process are 'nonspecific' because they occur before any crystals are present, and simply arise from the ion binding affinities and hydration capacity of the negatively charged polymer. The nonspecificity of the PILP process is reinforced by the fact that other nonstructured additives, such as polyacrylic acid, work just as well. Based on our *in vitro* studies using a variety of polyanion additives and several different

mineral systems, we have proposed that the acidic macromolecules involved in biomineralization should be considered as more than simply inhibitors or promoters, or crystal growth modifiers, but rather as process-directing agents. Our goal is to further understand the function of these additives/impurities, which we believe may be paramount towards understanding biomineralization.

4.2 BONE

4.2.1 THE STRUCTURE OF BONE

Bone is a classic example of the hierarchical levels of structure found in many biological tissues [115]. A review article by Weiner and Wagner [116] breaks down the structure of bone into seven levels of hierarchy (Figure 4.10); starting with self-assembled collagen fibrils (Level 1), in which nanoscopic platelets of hydroxyapatite (HA) are oriented and aligned within the collagen fibrils (Level 2); the collagen fibrils are layered in parallel arrangement within lamellae (Level 3); the lamellae are arranged concentrically around blood vessels, forming osteons (Levels 4 and 5); and finally the osteonal bone is either packed densely into compact bone, or is composed of a trabecular network of porous bone, referred to as spongy or cancellous bone (Level 6). At the macroscopic level, bone is a living tissue in which the cells organize and remodel their microstructure in response to external forces, compensate for holes and empty columns (areas of classical stress concentration in solid structures), and repair and regenerate the tissue if damaged [117].

Many researchers have contributed to the characterization of this complex tissue [118–125], for which there has been considerable controversy over its structure throughout the years. Studies on the mineralization of turkey tendon have provided important information at the primary level regarding the location of HA crystallites in the collagen fibrils of bone (note, turkey tendon naturally mineralizes, and has been utilized as a model of intrafibrillar bone structure) [121–126]. Figure 4.11 shows illustrations taken from the classic work of Weiner and Traub of a mineralized collagen fibril from turkey tendon and a schematic of the classic ‘deck-of-cards’ arrangement of the nanocrystals within the fibril [126]. The crystals are both physically aligned with the long axis of the collagen fibril, and crystallographically oriented with their *c*-axes (the direction) parallel to the fiber axis. This most fundamental level of bone, its nanostructured architecture, is a result of *intrafibrillar* mineralization, where the nanocrystals of hydroxyapatite are formed within the interstices of the collagen fibrils, as well as between fibrils (i.e. interfibrillar). According to Martin *et al.*’s *Skeletal Tissue Mechanics* [117], whole bone from dogs contains around 43 % by weight HA, with 58 % of the mineral being intrafibrillar, 14 % interfibrillar and 28 % in the gaps between collagen ends.

The term intrafibrillar is used here to specify the location of the crystallites, which Weiner, Traub and Arad [127,128] describe as a uniaxial parallel alignment of nanocrystals that are embedded within the gaps and grooves of the assembled

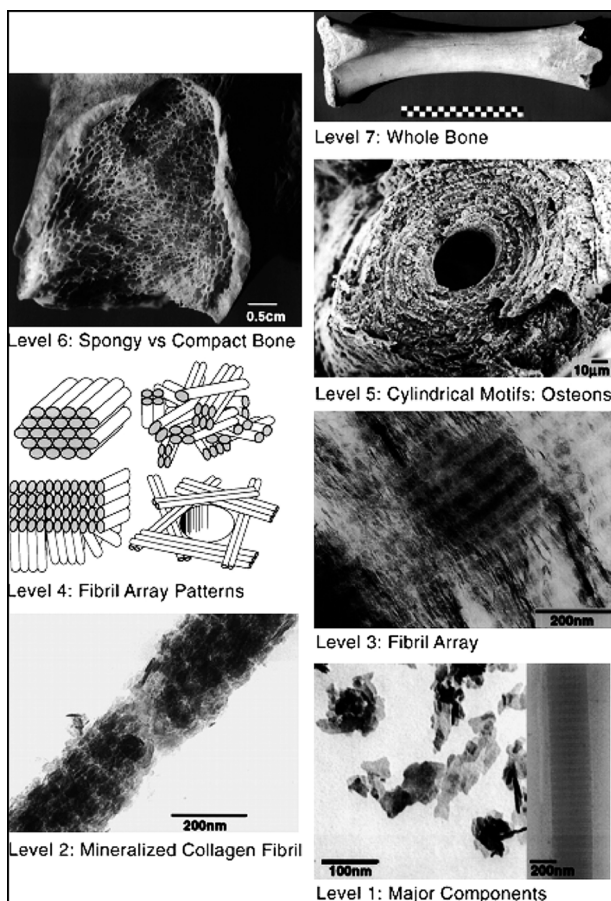


Figure 4.10 Seven levels of hierarchical structure of mammalian bone, as described by Weiner and Wagner. Level 1: Major components of bone, type I collagen and hydroxyapatite (HA). Note the native 64 nm banding pattern of type I collagen. Bars = 100 nm (left), 200 nm (right). Level 2: Intrafibrillar mineralization of type I collagen with nanoscopic platy hydroxyapatite crystals. Bar = 200 nm. Level 3: Fibrillar array of intrafibrillarly mineralized collagen. Note how the banding pattern of the collagen fibrils aligns across adjacent fibrils, suggesting that the collagen is congruently aligned across fibrils. Bar = 200 nm. Level 4: Fibrillar array patterns of mineralized collagen have a gradual splay in orientation across the lamellae, which provides better mechanical properties in all directions. Level 5: Concentric lamellae are arranged around canals as cylindrical motifs called osteons. Note the Haversian canal in the middle of the osteon, used to supply blood and cells to bone tissue. Level 6: Spongy vs. compact bone. Spongy (cancellous) bone is a continuous network of mineralized trabeculae, resembling a sponge, which optimizes the strength to weight ratio of bone, and helps absorb mechanical shock. Compact (cortical) bone is comprised of osteons packed tightly to provide mechanical strength and stiffness, and is observed on the outer case of bone and in the shafts of long bones. Level 7: This level consists of a range of all the bones in the body (e.g. flat vs long bones). (Reprinted, with permission, from the Annual Review of Materials Science, Volume 28 © 1998 by Annual Reviews www.annualreviews.org from Weiner S, Wagner HD, The Material Bone: Structure Mechanical Function Relations, *Annual Review of Materials Science* 28: 271–298 (1998)).

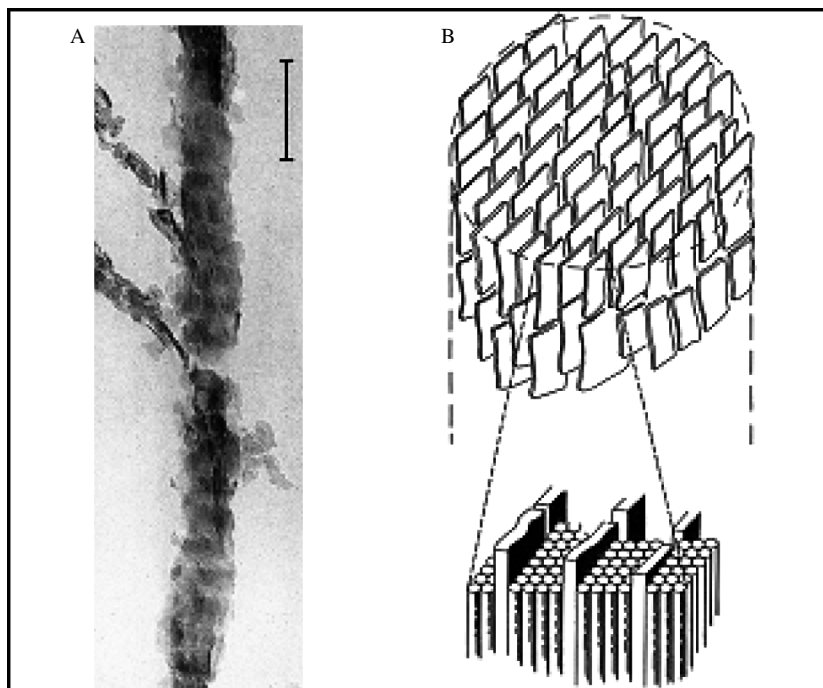


Figure 4.11 Figures reprinted from the classic paper by Weiner and Traub, showing the 'deck-of-cards' arrangement of crystallites within a collagen fibril. (A) TEM micrograph of mineralized collagen fibril from turkey leg tendon, showing layers of platy HA crystals. The banding pattern is thought to be due to more mineral being present in the gap regions of the collagen than in the overlap regions. The fibril is unstained and embedded in a thin layer of vitreous ice. Bar = 200 nm. (B) Schematic illustration demonstrating that the plate-shaped crystals are all uniaxially oriented with their *c*-axes parallel to the longitudinal axis of the fibril. The crystals are arranged in parallel coplanar arrays, and are thought to form within grooves across the fibrils. The grooves are separated by four layers of triple helical tropocollagen units (not drawn to scale). (Reproduced with kind permission of Springer Science and Business Media from Weiner S, Traub W, Organization of Crystals in Bone in *Mechanisms and Phylogeny of Mineralization* (ed. Suga S and Nakahara H), 247–253, Springer-Verlag, New York (1991).)

collagen fibrils (Figure 4.11). These gaps are created by the staggered arrangement of tropocollagen molecules (triple helical rods), which leads to periodicity of the hole and overlap zones. An associated periodic contrast pattern is commonly observed by transmission electron microscopy (TEM) of collagen fibers [123,129–131] after the collagen has been stained with an organometallic, such as phosphotungstic acid.

Landis *et al.* [121,132] have shown evidence, from high-voltage TEM tomographic imaging of naturally mineralizing turkey tendon, that the hydroxyapatite crystals first appear within the hole zones of collagen (Figure 4.12). Then the dark electron-opaque region spreads throughout the fibrils to the point where the crystals overlap, finally forming bridges between crystals that appear to

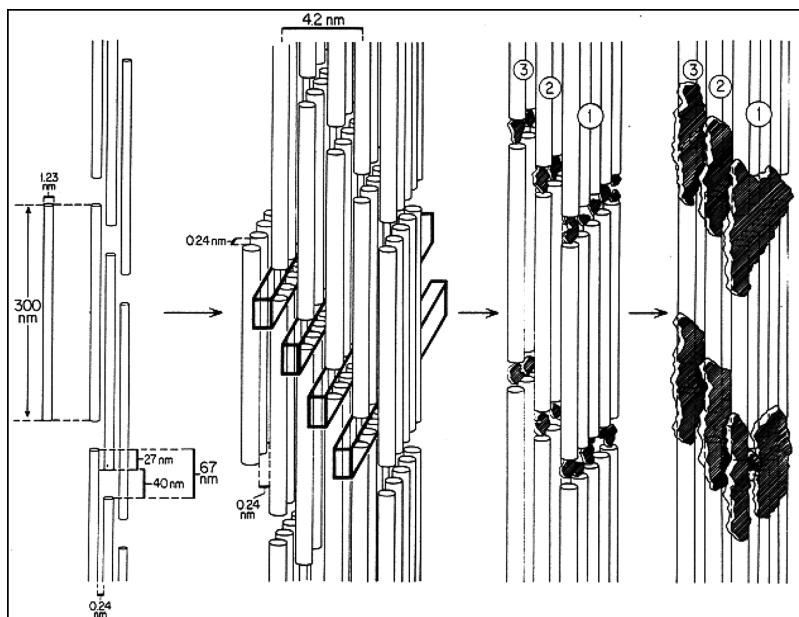


Figure 4.12 Schematic from Landis and coworkers, illustrating their *ex situ* TEM observations of mineralizing turkey tendon. Their studies support the prevailing theory on intrafibrillar mineralization of collagen in bone arising from specific nucleation domains (or proteins) within the collagen. The collagen self assembles into a quarter-staggered structure providing 40 nm gaps, which subsequently aligns to form grooves (rectangular boxes) between the tropocollagen units (cylinders). They observed that HA nucleates within the gap zones and continues to grow along the fibrils until fusing with like growing zones. (Reprinted from *Journal of Structural Biology*, Vol. 110, Landis WJ, Song MJ, Leith A, McEwen L, McEwen BF, Mineral and Organic Matrix Interaction in Normally Calcifying Tendon Visualized in 3 Dimensions by High-Voltage Electron Microscopic Tomography and Graphic Image Reconstruction, 39–54, Copyright 1993 with permission from Elsevier.)

fuse together into larger crystals. It should be cautioned, however, that the schematics that are found in such literature (as well as the ‘deck of cards’ description), although useful for illustrating microscopic observations, can be somewhat misleading. Electron diffraction data shows that in reality, there is a significant degree of rotational disorder, as well as tilting disorder, among the nanocrystals. The tilting disorder has been mentioned in the literature since the disorder is clearly indicated by the arcing of the diffraction spots. But the rotational disorder is not so obvious until one carefully scrutinizes the combination of spots/arcs that are present (see discussion in the section on *Intrafibrillar Mineralization of Collagen with Calcium Phosphate*). Nevertheless, the roughly parallel arrangement of the nanocrystals is clearly directed by the collagen fibrils within which the crystals have formed, and this arrangement ultimately gives bone its unique mechanical properties.

4.2.2 THE PROPERTIES OF BONE

From a materials engineering perspective, it is the nanostructured architecture of bone that is intriguing, and makes the material quite difficult to define. For example, is bone a polymer-fiber-reinforced ceramic-matrix composite; or is it a ceramic-nanoparticle-reinforced polymer-matrix composite? In other words, the two interpenetrating phases are so intimately linked that mechanical properties of bone are distinctly different from ceramics or polymers, and therefore are difficult to reproduce. For example, the modulus (stiffness) of bone lies somewhere between traditional ceramics and polymers, with values reported in the range of 0.34–13.8 GPa [133], which is very broad due to the variability among people (and animals), and even the types of bone within one person. It is highly desirable to prepare synthetic bone graft substitutes with matching modulus to avoid problems of stress shielding. Stress shielding arises when a material stiffer than bone (such as a metal or ceramic) takes up all the mechanical load, such that local osteocytes sense that there is no stress on the bone, and respond by resorbing the bone surrounding the implant, according to Wolff's law (cells optimize the strength to weight ratio of bone tissue). This presents an immense challenge given both the difficulty in reproducing the mechanical properties of bone with synthetic materials, as well as the variability in properties of natural bone among different patients.

In comparison to the CaCO_3 tissues of invertebrates, which often times have less than 5 % organic matrix occluded within the CaCO_3 biomineral, bone contains a relatively large amount of organic matrix (approx. 30–40 wt % collagen). The high degree of mineral loading that is achieved by intrafibrillar mineralization leads to around 50–60 wt % mineral phase, with another 10 wt % of the biocomposite being composed of water and noncollagenous proteins. Even though water is a minor constituent, its significance should not be overlooked, because it contributes to the overall toughness of the biocomposite, acting something like a plasticizer. Certainly collagen has very different properties when imbibed with water vs. in the dehydrated state, and it is therefore critical to carry our mechanical testing on fresh bone in an aqueous based environment.

The nanostructured architecture of bone also plays an important role in its strength and fracture toughness [126,134–136]. The brittle ceramic phase, which is embedded within the energy absorbing polymer matrix, helps mitigate crack propagation and fracture. The superior strength of bone and other biocomposites (e.g. nacre and teeth) stems from the nanometer size of the mineral particles, which ensures optimal strength and maximum tolerance to flaws [137]. Heavy loads can be carried by the high volume fraction of mineral platelets, whereas the protein transfers load via the high shear zones between the mineral platelets.

Lastly, the nanoscale dimensions of HA is an essential feature of bone in terms of its bioresorptive potential. Although bone is usually thought of in terms of its skeletal support function, it also provides an important metabolic function. An easily accessible means for resorption and deposition of this mineralized tissue is needed to carefully regulate ion concentrations, which although needed by cells, can be

harmful at excessive concentrations (this tight regulation of ion concentration is true of all biological systems, and most likely lies at the evolutionary foundation of regulating biomineral deposition). Because of the low K_{sp} of hydroxyapatite, large crystals of HA are only sparingly soluble when placed in the physiological medium. This is seen by the poor resorbability of the multitude of bone graft substitutes that have attempted to use HA in their formulations. More recently, researchers have had more promising results by preparing bone graft substitutes as composites with nanoparticles of HA (or metastable CaP phases) to enable osteoclast resorption for regeneration of bone tissue [133]. Clearly one cannot assume that because HA is a natural ingredient of bone, that it will be bioresorbable; it also requires the proper nanostructure, as in bone.

4.2.3 THE ORGANIC MATRIX OF BONE

As is true of many biominerals, the organic matrix not only influences the mechanical properties, generating bioceramic composites with both unique structures and properties, but it also plays an important role in regulating the formation of the composite. The collagen matrix is central to both aspects in the case of bone formation. There are many different types of collagen, but secondary (osteonal) bone consists primarily of type I collagen, which assembles into fibrils approximately 80–100 nm in diameter. While the type I collagen matrix is not the only factor in the formation of bone tissue (e.g. type I collagen is found in other tissues that do not mineralize), its organization and chemical structure are clearly central to understanding how secondary bone is formed. Therefore, there is a vast amount of information in the literature regarding the structure of collagen in mineralizing tissues.

The first widely accepted model for the organization of the collagen molecules was proposed by Hodge and Petruska [123], who described the quarter staggered arrangement of collagen's triple helices (tropocollagen units) which results in hole and overlap zones. Such overlap zones lead to electron dense regions, which can be observed as a periodic banding pattern in TEM of stained tissues, and more recently by AFM. A variety of modifications to this quarter staggered model have also been proposed, such as the alignment of gaps to form grooves (Figure 4.12), which are proposed to help account for the fact that the dimensions of the HA crystals extracted from bone are larger than the dimensions of the gap zones where they presumably are formed. Clearly before one can gain a better understanding of how intrafibrillar mineralization occurs, there needs to be greater advances in the analysis and organization of the organic matrix, both before and after mineralization. Much of the current data stems from demineralized tissues, which is inherently problematic due to changes in the organization and dimension of the original organic matrix after the demineralization process.

Close to the mineralization front, there are also noncollagenous proteins (NCPs), many of which are acidic macromolecules, and are thought to play an important

role in the mineralization process ([125,138–140]. Therefore, many studies have focused on determining the nucleating or inhibitory activity of NCPs on HA. There is a huge body of literature in this area, but we will not attempt to provide a detailed description of the purported role(s) of the various NCPs. Instead, it is hoped that at this point, when the reader comes across statements such as this, the thought should enter his/her mind that perhaps these inhibitory proteins are playing the role of process directing agents. We certainly believe this to be the case, as will be described in the section on *Mineralization of Collagen with the PILP Process*.

4.2.4 THE MINERAL PHASE OF BONE

The composition of the mineral substance in bone is described as consisting of poorly crystalline, nonstoichiometric (calcium deficient) hydroxyapatite, $\text{Ca}_{10}(\text{OH})_2(\text{PO}_4)_6$, which usually also contains varying degrees of carbonate (called carbonated hydroxyapatite or dahllite), magnesium and other substitutions [141]. Even the degree of hydroxylation in HA has been debated [142]. Early on, there was much debate as to the morphology of the HA crystals within bone, whether it was comprised of platelet-shaped, or needle-, filament- or ribbon-like crystals [131,143–147]. It has become generally accepted that the needle-like appearance was due to the edge on view of very thin platelets, but for some time, the actual location of the crystals remained uncertain. Using TEM stereomicrographs, Weiner *et al.* [118] demonstrated that the crystals were plate-shaped crystals and were preferentially located at the gap zones of the collagen, being stacked much like a deck of cards. The three-dimensional high voltage tomographic reconstructions of naturally mineralizing turkey tendon by Landis *et al.* [132,148] also demonstrate that the HA is primarily platelet-shaped crystals.

The size of bone crystals reported in the literature varies, with values ranging from length: 30–50 nm; width: 15–30 nm; and thickness: 2–10 nm [131,146,147]. This is in part due to sample preparation, but more importantly, the type of mineralized tissue (e.g. animal species, maturation and location of the tissue examined). In the case of mineralizing turkey tendon, Landis *et al.* [132] found that crystal widths varied from 30–45 nm, but thickness was uniform at $\sim 4\text{--}6\text{ nm}$. Such extremely small crystallites, of only a few unit cells in thickness, would not normally be thermodynamically stable, and evidently are stabilized by their intimate association with the organic matrix. Presumably, the crystals become unstable once the collagen is removed (such as by osteoclast secretion of enzymes), enabling the biological resorption of bone. Bioresorption differs from biodegradation, because it is an active process controlled by the cells, enabling the resorption to be carried out in concert with the rebuilding of bone by osteoblasts. Although there has been a strong push in the hard tissue biomaterials community to make biodegradable bone prosthetics, this seems like a risky prospect, because it is hard to envision that the degradation rate could ever be precisely matched to the regeneration of new bone (which is so variable among different patients, and different locations of the bone). We believe

that the only viable approach to a load-bearing bioresorbable bone graft substitute will require a material that the cells recognize as being like bone, and can resorb and remodel the material as they do in the natural process of bone regeneration.

4.2.5 FORMATION OF BONE

The mechanism of bone formation differs substantially between primary and secondary osteogenesis [149]. Epiphyseal cartilage, which serves as the basis for primary bone formation, is a combination of ground substance (proteoglycans and water), and loose, randomly arranged fibrillar bundles of collagen. The collagen fibrils are small (10–20 nm in diameter), and primarily type II collagen, although some type I can be present (e.g. $\sim 17\%$ type I in rat cartilage) [150]. There is a high occurrence of matrix vesicles, which are believed to deliver either a high concentration of ions to the mineralization front [151], or crystals which serve as the nidus for further crystallization [133,151]. The earliest mineral forms in the ground substance as clusters of HA crystals, sometimes referred to as calcospherites or calcification nodules [151]. As the metaphysis is approached, the clusters grow larger until they have formed a calcified cylinder around the chondrocyte cells. The mineralization is relatively rapid and unorganized, forming a woven bone microstructure. Although collagen is present in this ground substance, it is not organized into lamellae, as occurs when osteoblasts secrete collagen during secondary bone formation. Cameron suggested that the collagen fibrils found in cartilage are too narrow for the mineral to deposit within them, thereby resulting in the observed interfibrillar mineralization [150]. In this instance, the collagen does not appear to play an appreciable role in directing the mineralization process, and therefore will not be the focus of this review.

In secondary bone formation, primary bone is remodeled into the more mechanically robust structure of osteonal bone (in humans; not all animals have the same bone structure). Because of the difficulty in isolating secondary bone formation from the already present primary bone, naturally mineralizing turkey tendon serves as a useful model system of intrafibrillar mineralization, in which primary deposition of mineral is observed in the hole zones [119,125,152,153], as is the case for secondary bone. The collagen fibers are well organized, being aligned parallel in the tendon (but not as concentric lamellae as in osteonal bone). The timing of mineralization occurs at around 8 weeks of age in turkey, and therefore one can examine the process *ex situ* to catch a glimpse of the stages of mineralization. Landis has capitalized on this feature in his tomographic imaging studies of mineral formation in turkey tendon, which provides a three-dimensional reconstruction of the location and shape of the crystals within regions of newly mineralized tendon [154].

In both turkey tendon and secondary bone, the mineralization process appears to be directed by the collagen, which can be seen by the organization of the crystals which are intimately associated with the collagen scaffold [125]. The collagen fibrils, which assemble after secretion by the osteoblasts, are larger than those in

primary bone (20–80 nm in diameter), and assemble into a close packed lamellar structure. Exactly how this extracellular self-assembly into lamellar structures occurs is apparently unknown, but involves enzymatic cleavage of the telopeptide ends of the individual procollagen molecules, rendering them as tropocollagen units which then organize into the staggered array of fibrils. Reconstituted collagen, due to its liquid crystalline nature, will assemble into fibrillar structures *in vitro*, but how the fibrillar units are then organized into close packed parallel arrays *in vivo* (after being secreted from the cell) is still a mystery. Although considerable effort has gone into studying this amazing multifunctional protein, there are still many pieces of the puzzle that remain to be solved.

Protein–Mineral Interactions in Bone Formation

Two opposing themes can be found in the literature regarding collagen associated mineralization in secondary bone formation. Our work can possibly provide a bridge between these opposing views. The prevailing theory, first proposed by Glimcher and later supported by Weiner *et al.* and Landis *et al.*, is that the crystals nucleate within the hole zones of collagen and subsequently grow from the primary nuclei until fusing with crystals from neighboring zones [128,131,132,153,155]. An alternative view argues that initially, an amorphous ‘inorganic substance in bands’ is deposited within the hole zones (Figure 4.13A) [125,132,144,156,157], followed by crystallization into HA. We have also observed amorphous mineral in the collagen fibrils of bone (Figure 4.13B). In our case, the specimen was from a mature equine bone, so it is not clear if this particular region was being remodeled, or if some bone never fully crystallized. In either case, it does seem to support the hypothesis that the collagen in bone is mineralized with an amorphous precursor phase.

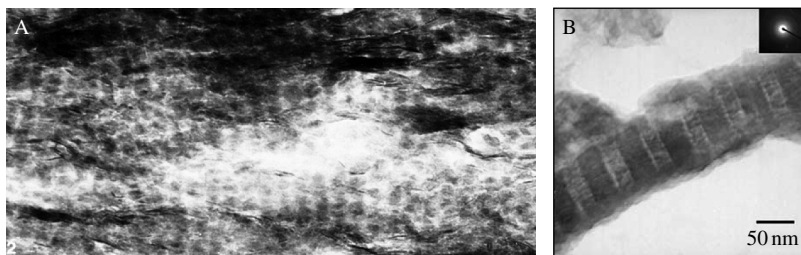


Figure 4.13 Evidence supporting an amorphous precursor in bone. (A) TEM images from Bonucci, show an ‘inorganic substance in bands’ during the early stage of bone formation. As the material crystallizes, it transforms into dark streaks (such as those seen at the top), which are platelets of HA embedded within the fibrils. (Reproduced by permission of CRC Press from Bonucci E, Role of Collagen Fibrils in Calcification in *Calcification in Biological Systems* (ed. Bonucci E), CRC Press, Boca Raton, 19–39 (1992).) (B) We have also observed a bone specimen that contains amorphous mineral. In this equine bone specimen, the sample was not stained, so the pronounced contrast is from an electron dense mineral phase only, yet no diffraction peaks could be found with selected area electron diffraction, demonstrating that the mineral is amorphous.

On the other hand, the alternative hypothesis is also strongly supported in the literature. In this hypothesis, the insoluble organic substrate presumably templates the nucleation event, such as by spatial organization of ions interacting with functional groups of the matrix [21]. As previously mentioned, HA nuclei have been observed by TEM (a high vacuum technique) to first occur in the hole zones of the collagen [119,152,153,158,159]. Yet, the lattice mismatch between collagen and HA [160], as well as the relatively weak ability of collagen to bind Ca^{2+} ions, suggests that the collagen substrate itself would not provide a good template for directing the nucleation of HA. Indeed, this has been shown to be the case through *in vitro* studies, in which HA nucleates on the surface of type I collagen fibrils [161] in a rather loose and nonspecific fashion (Figure 4.14), and only on the surface (not intrafibrillar).

In order to account for this inability of collagen alone to preferentially nucleate HA within the hole zones, theories have now turned towards the NCPs, many of which are phosphorylated proteins (e.g. osteonectin, osteocalcin, osteopontin), and are observed to be in close association with the mineralization front [162,163]. These proteins, which bind Ca^{2+} and have high affinity for collagen, are believed to adsorb to collagen and sit within the hole zones [162] to promote the nucleation of HA. Although collagen binding (at the surface of the fibrils) and Ca^{2+} binding have been demonstrated separately, the ability to nucleate HA in an intrafibrillar fashion has not been demonstrated *in vitro*. Therefore, in light of what we now know can be accomplished with Ca^{2+} binding proteins, our lab has recently proposed that the role of some of these NCPs might be as process directing agents. How this would

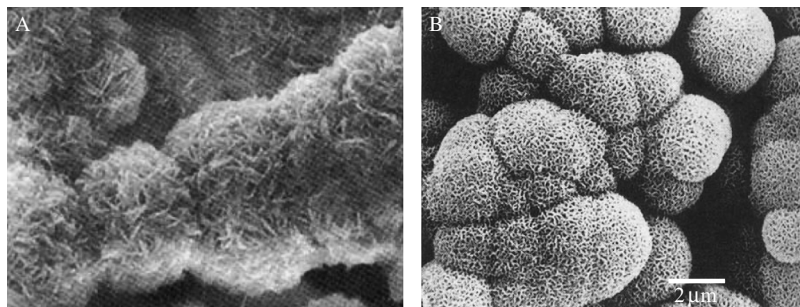


Figure 4.14 Literature examples of hydroxyapatite nucleation on collagen fibrils. When collagen substrates were introduced into simulated body fluid (SBF), the ‘bone-like’ apatite grew into spherulitic clusters, and appears to have only nucleated on the surface of the fibrils. (A) Spherulitic β -type hydroxyapatite nucleated on reconstituted type I bovine collagen fibrils placed in SBF. No scale bar indicated in publication. (Reproduced by permission of The Chemical Society of Japan from Giriya EK, Yokogawa Y, Nagata F, Bone-like Apatite Formation on Collagen Fibrils by Biomimetic Method, *Chemistry Letters*, 702 (Jul 5, 2002).) (B) Hydroxyapatite spherulites which nucleated on a collagen sponge. Bar = 2 μm . (Reproduced by permission of Blackwell Publishing Inc. from Rhee SH, Tanaka J.: Hydroxyapatite Coating on a Collagen Membrane by a Biomimetic Method, *Journal of the American Ceramic Society*, 81: 3029 (1998)).

enable intrafibrillar mineralization is presented below. But first, it will be instructive to present the more traditional view for comparison.

Proposed mechanisms for protein regulation of mineral formation generally fall into two categories, which can be considered to rely on ‘specific’ vs ‘non-specific’ interactions. The first model considers the protein to have a specific structure (such as domains with α -helical or β -sheet folding) which places charged groups with a periodicity roughly matching the spacing between ions for a particular crystal face of the mineral. This fit, or lattice matching, of solution ions to the charged groups in the protein could serve to promote nucleation. This model is commonly referred to as epitaxy (or pseudoeptaxy, since true epitaxy cannot occur between an organic and inorganic). For example, using a steady state gel system, Hunter *et al.* established that bone sialoprotein is one of the few matrix proteins that truly promotes rather than inhibits mineralization of HA [164]. Osteocalcin has also been found to accelerate nucleation, yet it specifically binds to the (001) plane, suppressing crystal growth perpendicular to this plane [165].

It is difficult in such systems to prove if epitaxy is really at play, or if there are less specific interactions stemming from interfacial energetics. For example, structural studies to date of bone Sialoprotein and related proteins in solution have found little inherent structure and crystallization remains elusive due to their anionic nature. Therefore, the latter mechanism may be more apropos. The fact that *in vitro* experiments are able to duplicate the bone like *c*-axis orientation of HA when precipitated in the presence of collagen fibrillogenesis (without using NCPs or other additives), suggests that interactions with the collagen alone provide sufficient ‘molecular recognition’ for nucleating crystallites (see following section). Yet, as mentioned above, HA precipitated on pre-assembled collagen fibrils does not seem to lead to this preferred orientation. Our research, as discussed below, supports the latter mechanism, and can provide some answers to this puzzling paradox.

In the second model, the specific arrangement of the charged groups on the protein is less critical; instead, the function of the protein is to sequester ions and increase the local concentration so that a critical nucleus of ions can be formed, leading to the formation of the mineral. One might consider this a nonspecific promoter of nucleation, which has been described by Crenshaw as ionotropic nucleation [166,167]. This latter model may seem similar to our description of the PILP process, and it is, but with several important differences. First of all, the PILP process is not induced by promotory proteins, but instead results from inhibitory proteins, because this inhibitory delay allows sufficient time to sequester ions while nucleation is inhibited within the localized highly supersaturated environment. This then leads to the second and more significant distinction, which is that the PILP process entails a physical phase transition. A distinct phase affords one the ability to manipulate its location, as well as the final morphology of the crystals, which as the reader may recall, retain the shape of the precursor. As we have discussed in our other papers, which deal more with the CaCO_3 PILP system, this capability can lead to mineral films (Figure 4.5D), fibers (Figure 4.7A), and molded crystals (Figure 4.7C).

These elaborate morphologies are not seemingly found in the CaP biominerals of vertebrates, so one might not anticipate such a process being relevant to CaP

biomineralization. But in fact, dental enamel is composed of fibrous ‘rods’ of CaP, which are ‘secreted’ in direct apposition to the Tomes process of ameloblast cells. We have shown that mineral ‘fibers’ can be ‘extruded’ by the PILP process (Figures 4.7A and B) [48,101]. We presently do not have data with CaP fibers; therefore, we will not speculate further about relevance to enamel. But with respect to bone, one might consider that the extremely small crystallites of HA basically fill up the interstices of the collagen fibrils with mineral, suggesting that the nanocrystals are perhaps ‘molded’ within the fibrils. They are not molded into the exquisite morphologies seen in CaCO_3 biominerals, but nevertheless, do have shapes that are not well faceted (as in classically grown crystals with equilibrium morphologies).

4.2.6 BIOMIMETIC METHODS FOR MINERALIZING COLLAGEN

There are two manners in which researchers have attempted to mineralize type I collagen in order to recreate intrafibrillar mineralization. The first is by using a pre-existing collagen substrate to induce CaP mineralization. The second is through mineralization of CaP in conjunction with fibrillogenesis. In natural bone formation, the type I organic matrix is first deposited and then subsequently mineralized; therefore, there have been many attempts at duplicating intrafibrillar mineralization using pre-existing collagen substrates [168–181]. Most often the mineralization is performed by placing a collagen substrate into simulated body fluid (SBF) for a certain amount of time in order to induce calcification [177–179,181–183]. Girija *et al.* [177] reported the synthesis of spherical β -type carbonate apatite deposits nucleating along the collagen fibrils (Figure 4.14A). On the other hand, Rhee and Tanaka [178] determined that the formation of carbonate apatite only occurred on collagen membranes soaked in SBF in the presence of citric acid, citing the strong chelating effect of citric acid on calcium ions (Figure 4.14B). In either case, the apatite mineral nucleates as spherulitic clusters on the collagen substrate. Often times these types of structures are referred to as bone like apatite because the platy appearance of the crystals resembles the nanoscopic platelets of natural bone apatite (very roughly), and are oriented in the [001] direction from the surface of the substrate. We find the use of this nomenclature to be misleading, because if it were to truly be bone like, the apatite crystals would be intrafibrillar, and the crystals would be parallel to the substrate (such as in collagen fibrils). In actuality, most people are only describing a common platy HA morphology that naturally grows in the [001] direction from many substrates that nucleate HA. It is not clear if the (001) plane is a common nucleation plane, or if the [001] direction is simply a fast growth direction that squeezes out other crystal orientations when polycrystals are crowded as they grow under high nucleation density conditions.

Researchers have also been testing the idea that the spatial charge distribution of organic macromolecular substrates, both collagenous and synthetic, can be used to induce oriented mineralization [184,185]. Stupp and coworkers demonstrated that by designing fibrous peptide amphiphiles (PA) with periodic, negatively

charged surfaces, thereby mimicking the charged NCPs adsorbed to collagen, they could induce hydroxyapatite crystals which were oriented with respect to the long axis of the PA fibril (Figures 4.15A and B) [184]. At least in this model, the HA crystals were aligned parallel to the peptide fibers; but how this system provides an understanding of intrafibrillar mineralization in bone is less clear. Goissis *et al.* [185] were able to form a hydroxyapatite coating on the surface of collagen fibrils which were modified through hydrolysis of asparagine and glutamine carboxyamide side chains to the acidic forms (Figure 4.15C). They claim that amide hydrolysis occurred preferentially near the hole and overlap zones, as suggested by the decreased interband distances of the polyanionic collagen, and the charged functionality increased the calcium binding sites at those regions. These studies suggest that surface modification of type I collagen can provide a means for promoting crystallization, which in the case of the natural environment, might result from either glycosylation of the collagen, or adsorption of NCPs. Although these studies are promising, they still have not produced both oriented and intrafibrillar crystals of HA in collagen; therefore, some researchers have attempted to nucleate HA during fibrillogenesis [186–193].

Recent research within the past decade has demonstrated that renaturing of collagen during the hydroxyapatite mineralization process produces an oriented mineral phase along the *c*-axis of the collagen fibrils. The first to utilize this process was Bradt *et al.* [188], and although they did not achieve oriented mineralization, they demonstrated that nanometer needle like hydroxyapatite was in close association with the gap zones of collagen during fibrillogenesis (Figure 4.16A). Rhee *et al.* [187] and Zhang *et al.* [191] have since used this

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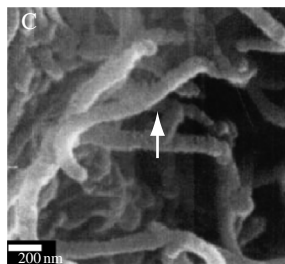


Figure 4.15 Electron micrographs of HA mineral deposited on functionalized polymer and collagen surfaces. (A) TEM micrograph of HA crystals (white arrows) mineralized on self assembled peptide amphiphiles. Bar = 20 nm. (B) Electron diffraction pattern taken from mineralized peptide amphiphile bundles demonstrating the presence of the (002) and (004) planes of HA. (Figures 4.15A and B are reprinted with permission from Hartgerink JD, Beniash E, Stupp SI, Self-assembly and Mineralization of Peptide-Amphiphile Nanofibers, *Science*, 294 (5547): 1684–1688. Copyright 2001 AAAS.) (C) SEM micrograph of functionalized collagen that has been mineralized with hydroxyapatite. The arrow points to mineralized ‘belts’ formed along the mineralized fibril. Bar = 200 nm. (Reproduced by permission of Blackwell Publishing Inc. from Goissis G, Maginador SVD, Martins VDA, Biomimetic Mineralization of Charged Collagen Matrices: In Vitro and In Vivo Study, *Artificial Organs*, 27 (5): 437–443 (2003).)

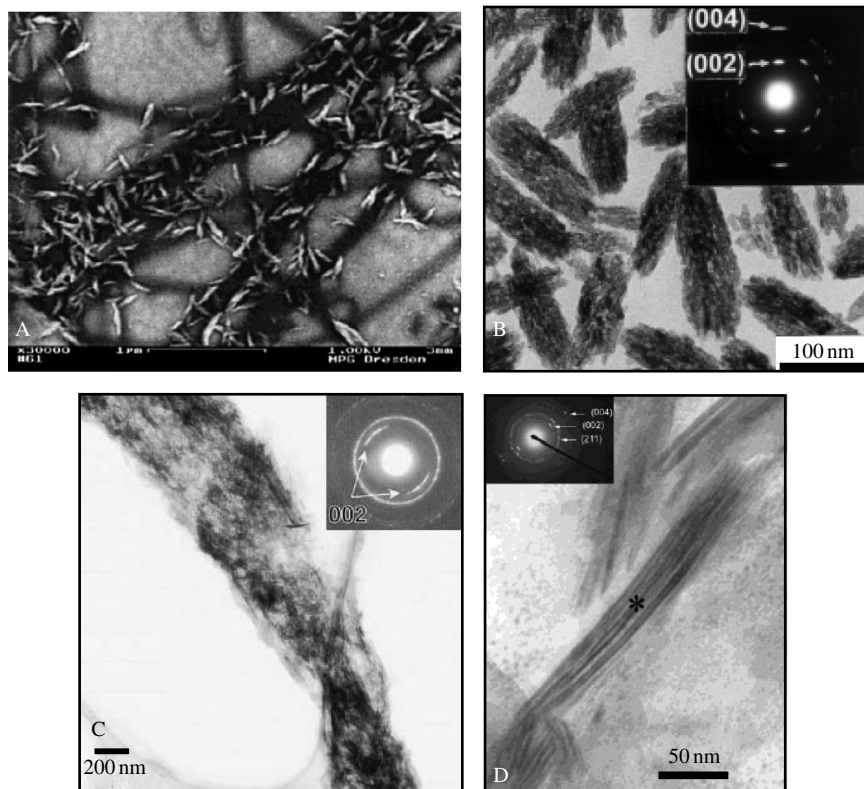


Figure 4.16 Mineralization of HA during fibrillogenesis of type I collagen. (A) SEM micrograph showing the presence of needle-like HA crystals in association with collagen fibrils, both transverse and parallel to the long axis. Bar = 1 μm . (Reprinted with permission from Bradt JH, Mertig M, Teresiak A, Pompe W, Biomimetic Mineralization of Collagen by Combined Fibril Assembly and Calcium Phosphate Formation, *Chemistry of Materials*, 11 (10): 2694–2701, Copyright 1999 American Chemical Society.) (B) TEM micrograph illustrating the oriented mineralization of HA formed during fibrillogenesis of type I collagen. The orientation of the crystals was determined by SAED (inset). Bar = 100 nm. (Reprinted from *Biomaterials*, Vol. 22, Rhee SH, Suetsugu Y, Tanaka J, Biomimetic Configurational Arrays of Hydroxyapatite Nanocrystals on Bio-organics, 2843–2847, Copyright 2001, with permission from Elsevier.) (C) TEM micrograph also illustrating the oriented mineralization of HA formed during fibrillogenesis of type I collagen. Note the arcing in the (002) planes in the SAED pattern (inset) indicating the mineral is not perfectly iso-oriented, which is true for bone as well. Bar = 200 nm. (Reprinted from *Biomaterials*, Vol. 22, Kikuchi M, Itoh S, Ichinose S, Shinomiya K, Tanaka J, Self-organization Mechanism in a Bone-like Hydroxyapatite/Collagen Nanocomposite Synthesized in vitro and its Biological Reaction in vivo, 1705–1711, Copyright 2001 with permission from Elsevier.) (D) Zhang *et al.* found crystallites of HA surrounding the collagen fibrils, which are thought to have aggregated into bundles of parallel arrays. (Reprinted with permission from Zhang W, Liao SS, Cui FZ, Hierarchical Self-assembly of Nano-fibrils in Mineralized Collagen, *Chemistry of Materials* 15: 3221–3226, Copyright 2003 American Chemical Society.)

approach to achieve aligned HA on collagen fibrils (Figure 4.16B), although with a different chemical reaction. Rhee *et al.* [187] added dissolved collagen to a phosphate solution and then mixed it with a calcium hydroxide solution. Interestingly, they showed arcing of the (002) plane in diffraction patterns of single mineralized fibrils, but no native banding pattern of collagen was observed. Similar results were observed by Kikuchi *et al.* [189] (Figure 4.16C), using a process similar to that of Rhee's. Zhang *et al.* [191] were also able to prepare oriented HA crystallites, which apparently wrapped around the fibrils (Figure 4.16D). They suggest the combined mineralization/fibrillogenesis procedure apparently stimulated aggregation and assembly of some of the mineralized fibrils into bundles. Strong interactions were seen to occur between the collagen and HA, as visually seen by HRTEM, and chemically demonstrated by peak shifts in the carbonyl bands, suggesting that such interactions are responsible for this bone like orientation.

While these results are quite promising for bone graft substitutes, they do not mimic the formation of naturally mineralizing collagen, in which collagen fibrils are already assembled prior to mineralization. In addition, it is not demonstrated that intrafibrillar mineralization has been achieved. The diffraction patterns in these studies indicate that there is oriented hydroxyapatite along the collagen fibrils, but the absence of electron dense banding patterns in their TEM data suggests that the mineral is only on the surface of the fibrils, and not intrafibrillar. This is perhaps not surprising considering that there is no intrafibrillar space prior to the fibers being formed, and in the case of bone formation, intrafibrillar crystallites are not created by aggregation and assembly of mineralizing units, since the collagen fibrils are already assembled prior to mineralization. Therefore, we find the significance of these studies (with respect to understanding bone formation) to be in how they demonstrate that a preferred crystallographic interaction exists between the chemical structure of collagen and the nucleating planes of HA. Interestingly, this preferred orientation is apparently not all that specific to collagen, given that a similar orientation is seen in all phosphatic biominerals, such as in dental enamel (through interactions of HA with the assembly of amelogenin proteins), and in the mollusk, *Lingula unguis*, (which is one of the few marine organisms to still use a phosphatic biomineral), where the HA crystals are aligned with their *c*-axes parallel to β -chitin fibrils [133,194].

Overall, these 'biomimetic' composite structures demonstrate that the scientific community is coming ever closer to creating bone graft substitutes that mimic the nanostructure of bone. Indeed, Kikuchi and coworkers [189] found that their composite structure was highly bioactive, being resorbed by osteoclasts and allowing subsequent osteoblast activity to build new bone. The next quantifiable leap in the preparation of an optimal synthetic material for a bone graft substitute would be to not only have a composition that matches bone, but to have load bearing capacity, as well as bioresorbability (cell regulated, and not biodegradation). In order to achieve this, we believe intrafibrillar mineralization will be the key component.

4.2.7 MINERAL PRECURSOR PHASES

While the final product of intrafibrillar mineralization in bone is oriented, platy HA crystals embedded within a type I collagen matrix, the initial phase observed within newly deposited collagen is considered by some to be amorphous [125]. There are even reports that suggest that bone apatite is paracrystalline, an intermediate between amorphous and crystalline calcium phosphate [195]. Recent research in the biomineralization field is pointing towards the use of amorphous phases in the formation of several calcium carbonate biominerals [52,67,69–71,196], but generally little connection between CaCO_3 and CaP biomineralization mechanisms are considered because their final morphologies are so distinctly different. But when considering the possibility of amorphous precursors, the relevant connection between these different systems has to do with how the precursor phase is manipulated, and not the final properties of the crystals.

The presence of CaP precursor phases in vertebrate biominerals has long been debated, both for octacalcium phosphate (OCP), and amorphous calcium phosphate (ACP). Precursor phases have been suggested to occur in the highly organized HA crystals formed within the collagenous matrices of bone and dentin [53], as well as the elongated prismatic morphology of HA crystals in dental enamel [125,197,198]. Posner was the first to propose that ACP is the initial precipitate in bone formation. This conclusion was based on the fact that HA nearly always forms from an amorphous precursor when precipitated inorganically. One has to go to very low supersaturation to avoid the kinetic pathway (Ostwald–Lussac Rule of Stages) leading to the formation of the more soluble ACP phase (unlike ACC, which is very unstable, and not usually seen without stabilizing additives). With respect to biological data, early bone is seen to have a lower degree of crystallinity than the more mature bone, as seen by a narrowing of the diffraction peaks with maturation. This indicates either an increase in crystal size or a higher degree of ordering.

Shortly thereafter, Bonucci supported this concept with *ex situ* evidence that a more electron dense, finely granular inorganic substance (with an amorphous appearance) is deposited in the hole zones of collagen during the first stage of secondary bone formation (Figure 4.13A) [53]. During the second stage, platy HA crystals are formed, eliminating any evidence of an initial amorphous phase (which is not easily detected). From our own experience, it is clear that the collagen fibrils that Bonucci imaged by TEM do contain some type of ‘inorganic substance in the bands’, because this distinct banding pattern cannot be seen in collagen which has not been stained. The collagen therefore must have contained some type of contrast agent (with higher atomic number), which in the case of natural bone, is evidently ACP.

In the case of dental enamel, the maturation is described as a slow transformation from a poorly crystalline HA to fairly well crystallized HA, yielding crystals that are much larger than those in bone or dentin [133]. In some cases, a stippled material has been seen during the early stages, which appears to arise from coalescence

of nanoparticles into ribbon shaped crystals, which later thicken to form the large crystals seen in enamel. Chemical force microscopy (which provides images based on lateral friction forces experienced as a functionalized AFM tip is dragged across a surface) finds that the rods taken from the maturation stage of enamel formation contain regularly spaced discrete domains of variable charge density [199]. This would not be expected if the crystals were to be formed by the classical nucleation and growth, in which an ion by ion deposition would be expected to give rise to a fairly uniform crystal surface. Instead, these various observations seem to support the notion that these rods are created by accumulation and aggregation of precursor particles (amorphous or paracrystalline), where each particle might have a slightly different chemical makeup, or additive content that gets excluded to the surface.

It has also been suggested that octacalcium phosphate (OCP) might act as a precursor to HA [200]. This is based on evidence of a ‘central dark line’ seen by TEM in biological apatites. This dark line, which is most prominent in HA rods of dental enamel, reportedly exhibits an OCP lattice. Because the atomic arrangements of the calcium and phosphate ion of OCP and HA are similar, with the only difference in crystal structure arising from the hydrated layers of water, it seems reasonable that an OCP precursor could easily transform into HA (Figure 4.4, pathway iii).

The amorphous precursor theories are based on the Ostwald–Lussac Rule of Stages, which as described earlier, finds that the most soluble (least stable) phase forms first during a sequential precipitation when the solution is supersaturated with respect to multiple phases. Because both ACP and OCP are known to be precursors to HA when precipitated *in vitro*, such studies support the concept that bone mineralization could occur via a metastable precursor mechanism. Of course, once the precursor phase is formed, crystals can then subsequently ‘nucleate’ from within the amorphous phase, and transform into the crystalline phase. In the case of amorphous precursor phases, this transformation could best be described as a pseudo-solid-state transformation, in which the thermodynamic driving force to reach the more stable crystalline structure drives off the waters of hydration as the ions are simultaneously reorganized into a crystalline lattice. Alternatively, crystallization from an amorphous precursor can occur via dissolution–reprecipitation. We have observed both mechanisms of crystallization from amorphous phases in our *in vitro* studies, and believe that in the case of bone formation, the pseudo-solid-state transformation seems most plausible.

At this point, we hope to have convinced the reader that some type of precursor mechanism could be relevant to how bones are formed, but the reader may still be wondering – how does the amorphous mineral precursor get *into* the collagen fibers, leading to *intrafibrillar* mineralization?

Mineralization of Collagen with the PILP Process

This is one of those places where we believe the concept of a *fluidic* amorphous precursor phase is important. We hypothesize that the nanoscopic gaps and grooves

of the collagen fibrils could give rise to capillary forces that would draw a fluidic precursor phase into the fibrils. Note, this capillary action occurs while the collagen is in solution, in the swollen state (it is not like a dry sponge). This is because large capillary forces can arise from curvature of a phase boundary (hence the importance of the phase boundary in the PILP process; where the role of the polymer is not just to chelate the ions). Once the fibrils are infiltrated with the amorphous precursor, it would solidify and crystallize, leaving the collagen embedded with nanoscopic crystals that are molded by the surrounding collagen matrix. Once again, this solidification occurs while the sample is still in the solution, and is due to the thermodynamic driving force to transform into the more stable crystalline phase. This new hypothesis on bone formation will be difficult to prove *in vivo*, but we have been successful in demonstrating proof of concept in our *in vitro* model system.

Considering that calcium phosphates naturally form an amorphous phase, the question may come to mind – how does the PILP phase differ from the amorphous CaP gel that commonly forms in CaP precipitations. This is where the business of differentiating between solid and liquid amorphous phases becomes tricky. At this point, all we can suggest is that the amorphous phase should be sufficiently fluidic to succumb to capillary forces, if our hypothesis on the mineralization mechanism proves correct. Clearly further work is needed to understand the stabilizing and structural differences between amorphous phases.

Intrafibrillar Mineralization of Collagen with Calcium Carbonate

In our first studies, we mineralized collagen with CaCO_3 PILP phase because at the time, we had not generated a CaP PILP phase, and were more interested in determining if the concept of mineralization via capillary forces was viable. Indeed, we found that collagen fibrils could be well infiltrated with calcium carbonate, leading to an interpenetrating composite of calcite and collagen [102,103,201]. More recently, we have achieved intrafibrillar mineralization with CaP, with even more exciting results [104].

In order to test our hypothesis that type I collagen could be intrafibrillarly mineralized via an amorphous liquid phase precursor, we used a CellagenTMsponge comprised of reconstituted type I bovine collagen as our substrate. The native 64 nm banding pattern of type I collagen, as seen in Figure 4.17A, was a clear indication that this was indeed fairly pure type I collagen. While this banding pattern can be observed using scanning electron microscopy (SEM), the same cannot be said for collagen examined using transmission electron microscopy (TEM) (Figure 4.17B). Since collagen is primarily carbon (C), there is no contrast mechanism (i.e. C has a low atomic number and therefore does not scatter electrons well), and thus the banding pattern is not visible. To overcome this problem, a heavy metal stain such as phosphotungstic acid (PTA) can be applied to the collagen in order to create contrast (Figure 4.17C). PTA, being a negative stain, occupies the holes zones and scatters electrons, leading to the dark banding pattern. From this experiment alone, it is apparent that, if an obvious banding pattern is observed within collagen in

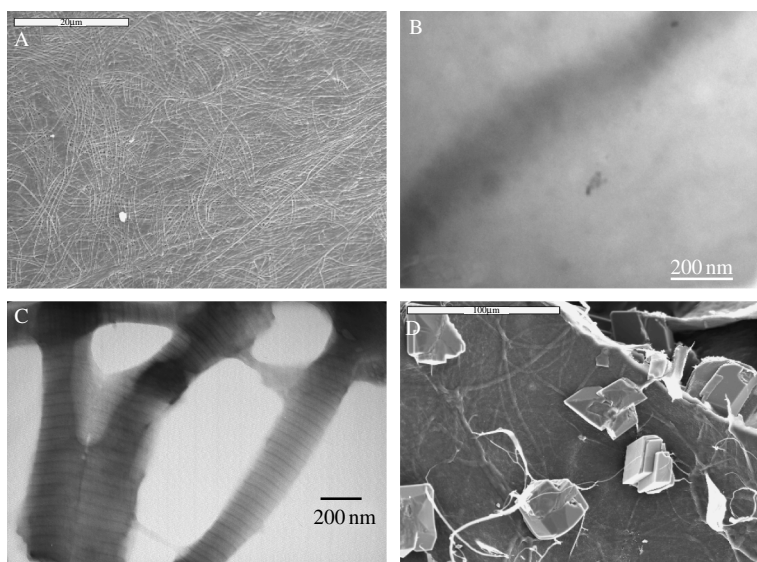


Figure 4.17 Electron micrographs of the bovine collagen sponge samples used in our experiments. (A) SEM of the Cellagen™ sponge, as received. The reconstituted type I collagen fibers are randomly organized and relatively loosely packed (as compared to the collagen fibers in tendon or lamellae of bone), and have diameters ranging from 250–670 nm. (Reproduced by permission of Olszta MJ, Douglas EP, Gower LB, *Intrafibrillar Mineralization of Collagen using a Liquid-Phase Mineral Precursor, Materials Research Society Symposium Proceedings*, 774: 127–134 (2003).) (B) Bright field TEM image of an unstained collagen fibril, demonstrating that the banding pattern can barely be discerned if no contrast agent is present. (C) Collagen fibrils strained with phosphotungstic acid (PTA) show the native banding pattern. The bands appear different than in bone, which does not exhibit such sharp lines, apparently because the crystals grow beyond the gap regions, whereas the PTA stain likely adsorbs at the interfaces. (Reproduced by permission of Olszta MJ, Douglas EP, Gower LB, *Intrafibrillar Mineralization of Collagen using a Liquid-Phase Mineral Precursor, Materials Research Society Symposium Proceedings*, 774: 127–134 (2003).) (D) The collagen sponge mineralized without polymeric additive (control reaction) only provided a substrate for heterogeneous nucleation, in which large (40–50 μm diameter) rhombohedral crystals of calcite nucleated randomly on the surface of the fibers.

TEM, there must be some contrast agent within the hole zones. The ‘contrast agent’ that provides the native banding pattern seen in *ex situ* examination of bone and naturally mineralizing turkey tendon arises from the electron dense mineral phase.

As has been mentioned earlier, crystallization in the absence of crystal growth modifiers yields crystals with faceted (equilibrium) morphologies. When a substrate is introduced, the crystals nucleate heterogeneously on the surface, if carried out at moderate supersaturation. This can be seen in our control reaction, in which type I collagen was placed in a simple crystallizing solution, without the polymeric process directing agent. Large crystals nucleated on the surface of the collagen (Figure 4.17D). Note, the large crystals are calcite, not hydroxyapatite, because as mentioned above, our preliminary work was done with the CaCO_3 system. Although

CaCO_3 is not the mineral constituent of bone, it was used here to demonstrate proof of concept that intrafibrillar mineralization could be achieved using a precursor process.

As minute amounts of acidic polymer (see referenced papers for details) were added to the crystallizing solution, and the supersaturation raised slowly, the mineralization of the collagen was markedly affected. When the mineralization was complete, only part of the CellagenTMsponge was mineralized due to the limited amount of PILP phase that is generated in a one batch reaction (Figure 4.18A). Interestingly, in the regions that were well mineralized, the fibrils retained the

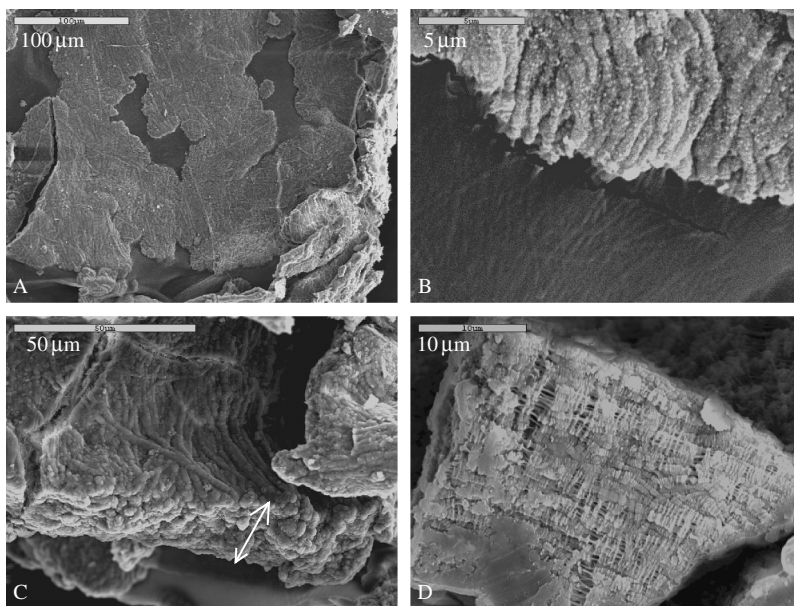


Figure 4.18 SEM micrographs representing the sequential PILP mineralization of the collagen sponge in the presence of polyacrylic acid. (A) After one mineralization step (a 3 day reaction), patchy calcitic films were deposited on the surface of the sponge. (B) A higher magnification view of the edge of one of the mineralized patches shows that individual subfibers are encased in mineral. Due to beam damage, a crack occurred in the region of the substrate that was not coated and protected by the mineral. There is likely high stress in this region due to the restraints applied by the solidifying mineral during dehydration (vacuum drying for SEM). (For Figures 4.18A and B, Copyright 2003 from *A New Paradigm for Biomineral Formation: Mineralization via an Amorphous Liquid-Phase Precursor*, *Connective Tissue Research* 44 (Suppl. 1): 326–334 (2003) by Olszta MJ, Odom DJ, Douglas EP, Gower LB. Reproduced by permission of Taylor & Francis Group, LLC., <http://www.taylorandfrancis.com>.) (C) After a second PILP mineralization (6 days), the amount of CaCO_3 deposited into the collagen sponge increased. This region shows that a fairly thick mineralized matt has formed (thickness indicated by arrow), which does not compact as much upon dehydration as the swollen organic sponge alone. (D) After five sequential mineralizations (15 days), the composite has become a rigid ‘brickette’ as the mineral precursor kept seeping into the structure, although a few regions remain open and porous.

dimensions of swollen collagen, while the unmineralized fibrils dehydrated back down to a much smaller diameter during the drying of the sample for SEM (Figure 4.18B). From the standpoint of an amorphous precursor infiltrating the interstices of the collagen, we envision the precursor displacing the water that had swollen the fibril, such that when the amorphous phase crystallized, it retained the shape of its container, in this case the swollen collagen fibrils. In order to more fully mineralize the sponge, it was subjected to sequential mineralizations by replenishing the solution with a fresh PILP crystallizing solution every 3 days. With each mineralization, the sample became more dense and thicker as the mineral held apart the collagen fibrils and allowed further infiltration of PILP phase (Figure 4.18C). After five mineralizations, the sample was nearly fully mineralized and quite rigid, and appeared to be a 'bricquette' of the collagen- CaCO_3 composite (Figure 4.18D).

To determine the extent of mineral infiltration into the collagen fibrils, we selectively etched away each of the components of the composite. A weak acid solution was used to dissolve the mineral phase (Figures 4.19A and B), while the organic collagen scaffold was removed by placing the mineralized samples in a dilute sodium hypochlorite (NaOCl) solution (Figures 4.19C, 4.19D and 4.20). In the acid etched samples, some mineral phase remained, appearing as discs that lie perpendicular to the fiber axes (Figures 4.19A and B). These discs may have been partially protected by the collagen fibers when they were in the swollen state (they are dehydrated in the SEM images, but would have been swollen during the etch treatment).

It was hard to tell from the SEM images in Figure 4.18 if the mineral had fully penetrated the fibrils, or if there was only a mineral coating surrounding the fibrils (Figure 4.19C). Upon examination of the deproteinized fibrils, it was found that the fibrils contained mineral throughout, and were not simply encapsulated with mineral. If it was merely a mineral coating, one would expect to see a hollow tube after removal of the collagen. Instead, the extent of mineral penetration was so high that the overall shape of the preexisting fibrous composite was maintained, and the discs of mineral phase can be seen to span the entire diameter of the preexisting fibrils (Figure 4.19D).

It was surprising that the CaCO_3 mineral appeared as discs, given that the HA crystals in bone are platy when they form within the fibrils. In the higher magnification views in Figure 4.20, mineral can be seen to span the diameter of these fiber bundles. In the fiber bundle shown in Figure 4.20B, the mineral appears to have a platy morphology, but they are much larger than the platelets of HA in bone. This could be related to the natural tendency of HA to form smaller crystals than calcite. However, if the mineral is fully extracted from the collagen scaffold, one does find the presence of nanoscopic platy crystals of CaCO_3 (Figure 4.21). So it appears that these discs are probably not single crystals of calcite, but are likely agglomerates of the nanocrystals. The X-ray data below supports this conclusion.

Although CaCO_3 was used to mineralize these samples, there were no clues, such as crystal facets, to indicate which phase was present in these composites. The control reaction, which was performed under similar conditions (except without

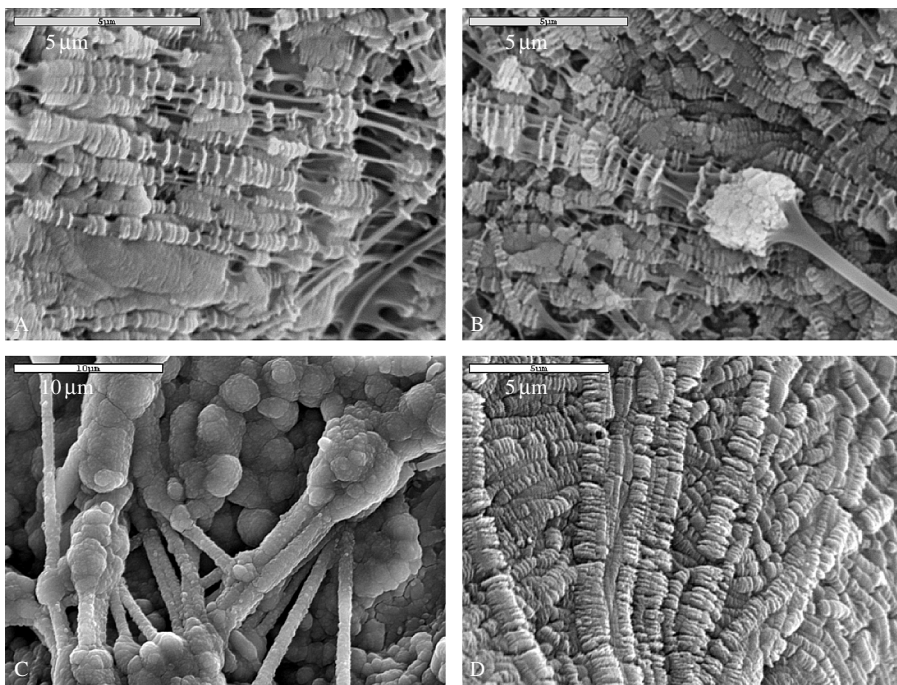


Figure 4.19 SEM micrographs of mineralized samples subjected to etching, for determination of the extent of mineral infiltration. (A and B) These mineralized collagen samples were treated with 0.1 M HCl for 15 minutes to remove excess surface coverage of mineral coating. (A) A roughly periodic banding pattern is presented by the calcite disks, which lie perpendicular to the *c*-axis of the collagen fibers, suggesting that excess mineral is present within periodic zones within the fibrils. (B) The mineral phase was more fully removed by the weak acid in this region, but still there are bands of CaCO_3 that appear to have been protected by the collagen fibers when they were in the swollen state. (C and D) These mineralized collagen sponges were treated with dilute bleach solution (0.5 vol% NaOCl) for 15 minutes in order to remove the organic matrix. (C) Fully mineralized samples which have a complete CaCO_3 coating are either not exposed to the bleach due to encapsulation and protection by the CaCO_3 coating, or the removal of collagen within cannot be observed. (D) Surprisingly, after the bleach treatment, a coherent structure remained, which was comprised of disks of calcite that were evidently embedded within the preexisting fibers. If the mineral only encapsulated the fibrils, one would expect to see hollow tubes after removal of the collagen.

polymer) yielded calcite crystals. But given the nondescript appearance of the mineral (which is typical of PILP formed crystals), or the disc like structures in the fibrils, it was not even clear if the mineral phase was amorphous or crystalline. X-ray diffraction (XRD) was used to answer these questions (Figure 4.21), and to illustrate the difference between nonmineralized collagen, the control mineralization reaction, and the PILP mineralized sample. From Figure 4.21A, it can be seen that the isotropic mat of collagen alone does not lead to sharp Bragg diffraction peaks (in a powder diffractometer), signified by the broad amorphous peak at lower

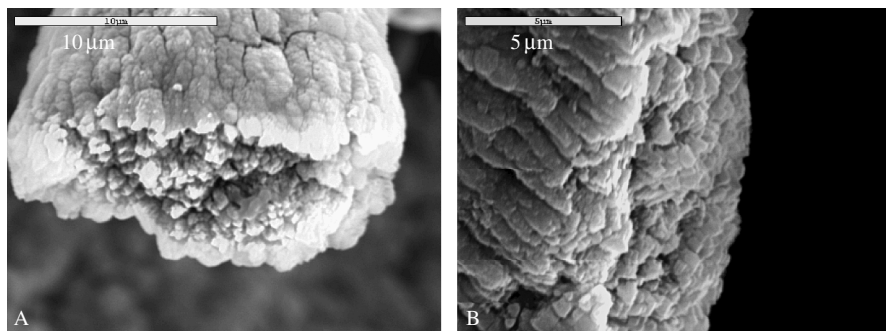


Figure 4.20 SEM micrographs of mineralized collagen bundles that were treated with a dilute bleach solution for 15 minutes. (A) This cross sectional view of a large 18 μm diameter collagen bundle demonstrates the depth of mineral penetration into the preexisting fibers. In this case, a banding pattern is not apparent, but instead, small crystallites appear to traverse completely across the bundle and link together the collagen subfibers. (B) The end of this fiber bundle shows aligned calcite crystals which exhibit a platy habit, similar to the ‘deck of cards’ arrangement described by Weiner and Traub for HA crystals in bone. However, the platy calcite crystals are much larger than the nanoscopic HA crystals in bone.

angles (commercial CellagenTM does not have parallel alignment, so fiber patterns are not seen). The control reaction nucleated calcite crystals on the surface of the collagen fibrils, which could be identified based on their rhombohedral morphology, but was verified by the sharp Bragg peaks (Figure 4.21B). The same peaks were obtained from the PILP mineralized sample (Figure 4.21C), indicating that the nondescript mineral observed by SEM was calcite. However, the peaks were more broad than those in the control reaction, which indicates that either the mineral is less crystalline, or made up of very small crystals. Figure 4.21D suggests that the broad peaks may result from the latter case.

At this point, we were pleased that we were able to demonstrate proof of concept that intrafibrillar mineralization of collagen could be achieved using the PILP process, even when using a mineral system that is not normally associated with collagen. There was obviously a high degree of uncertainty when attempting to duplicate a process using an unnatural mineral system, and especially if the mechanism relied on ‘specific’ protein–mineral interactions. Such a unique interpenetrating composite of collagen–calcite might be useful as a bone graft substitute, but given that such a material is not found in nature, one might ask, how is this relevant to understanding bone formation?

Intrafibrillar Mineralization of Collagen with Calcium Phosphate

Clearly to make this claim of relevance, we also needed to be able to achieve intrafibrillar mineralization of collagen with calcium phosphate, the true component of bone. After considerable effort in dealing with the more complicated CaP system, we can now confidently claim to have achieved this as well. The CellagenTM sponge

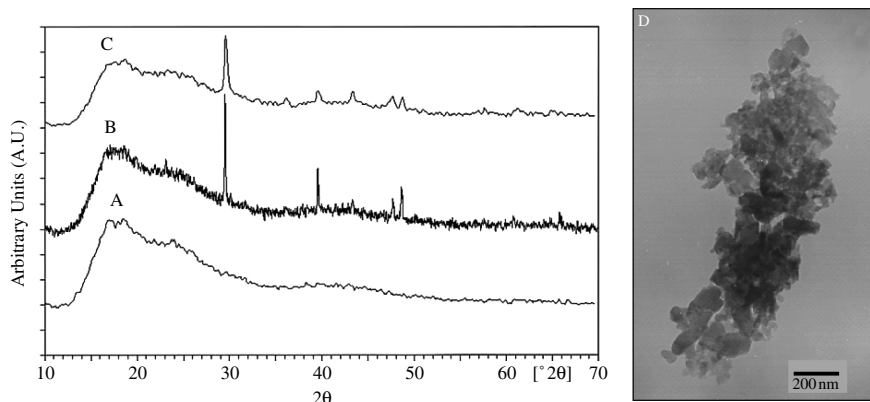


Figure 4.21 XRD spectra of the collagen sponge mineralized by different processes. (A) The XRD spectrum of the untreated sponge, corresponding to the sample imaged in the SEM micrograph of Figure 4.17A, shows a broad diffuse peak. This is expected since the organic matrix material is noncrystalline and isotropically oriented. (B) Spectrum B is of the collagen sponge mineralized by the direct crystallization route (without the polymeric process directing agent), corresponding to the SEM micrograph in Figure 4.17C. The large rhombohedral crystals observed in Figure 4.17C are clearly identified as calcite by the XRD peaks, especially the most intense peak at 29.5° corresponding to the (104) planes of calcite. (C) Spectrum C is of the collagen sponge mineralized with polyacrylic acid, corresponding to the SEM micrograph in Figure 4.20A. The XRD peaks are the same as those in spectrum B (except broader), indicating that the nondescript thin films encasing the collagen are in fact calcite. The broader peaks indicate that the mineral is either poorly crystalline, or is composed of very small crystals. (Figures 4.21A–C are reproduced by permission of Olszta MJ, Douglas EP, Gower LB, *Intrafibrillar Mineralization of Collagen using a Liquid-Phase Mineral Precursor*, *Materials Research Society Symposium Proceedings*, 774: 127–134 (2003).) (D) TEM of crystals extracted from the mineralized sponge support the latter, showing that the calcite crystals are very small and platy. This is somewhat surprising since the deproteinated samples (Figure 4.19) exhibited large discs, but evidently the discs are not single crystals of calcite, but agglomerates of nanocrystals.

was again used as the type I collagen scaffold for mineralization with CaP PILP phase. Figure 4.22A shows the TEM appearance of the collagen fibrils prior to mineralization (with PTA stain), for comparison to the image in Figure 4.22B of a mineralized fibril (without stain). The banding pattern can be seen, as well as striations, which we believe are nanocrystals. In this micrograph, one can also see droplets of the PILP phase adsorbed to the collagen fibril (arrows). SEM of the mineralized composite shows that the scaffold was more fully mineralized than the CaCO_3 composites, presumably because the lower solubility of CaP leads to more mineral precipitate (Figure 4.22C). The encapsulating mineral has a similar nondescript appearance to that seen for the CaCO_3 composites and bone (Figure 4.23), at least at the superficial level of examination. A high magnification view taken by field emission SEM shows what appears to be needle like crystals that run parallel to the long axis of the fibers (Figure 4.22D).

To support our hypothesis on the mechanism underlying this success, we examined the mineralization process in the early stages. As expected, the control

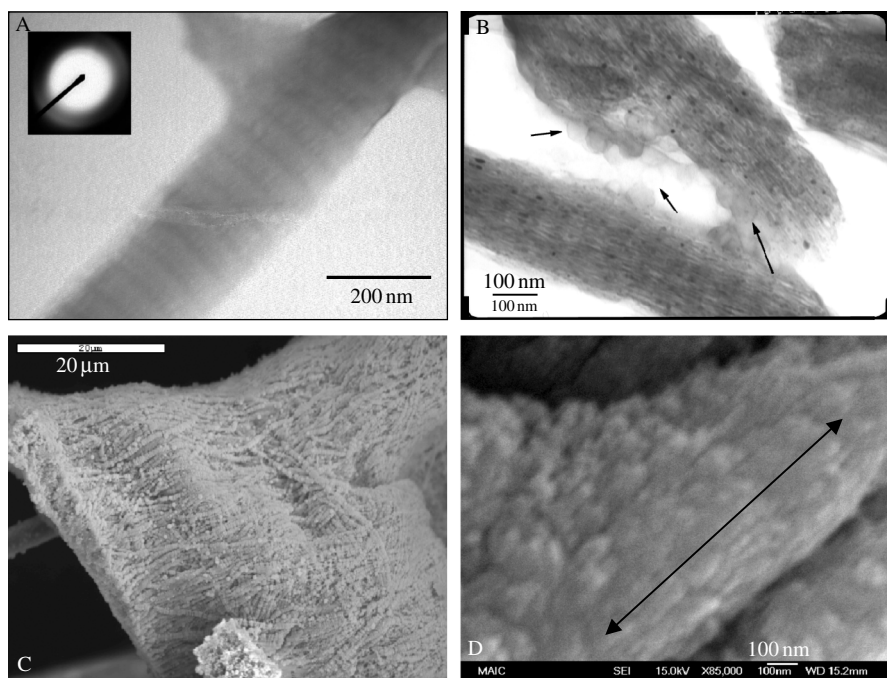


Figure 4.22 Mineralization of Collagen™ sponge with a CaP PILP phase. (A) Type I collagen fibers prior to mineralization show the periodic banding pattern of native collagen (with PTA stain). (B) The collagen fibers after mineralization show the periodic banding pattern from contrast of the mineral phase alone (no staining). Note, some PILP droplets have adsorbed to the fibrils (arrows). (C) The CaP system leads to a more fully mineralized sample than the CaCO_3 sample, presumably due to the lower solubility of HA, which leads to more precipitate. (D) High magnification view of the same sample using field emission SEM, showing needles of HA aligned with the collagen fiber axis (arrow).

reaction (without polymeric process directing agent) generated clusters of HA on the surface of the collagen (Figure 4.24A), which are similar to those reported in the literature (and shown in Figure 4.14). Clearly, the collagen alone does not stimulate crystal nucleation within the gap zones of the fibrils. When the same reaction is carried out under PILP conditions (with $15 \mu\text{g/ml}$ polyaspartic acid), an amorphous precursor is formed. This can be seen in an isolated collagen fibril examined at day one, which shows a distinct banding pattern in TEM, with a 64 nm periodicity (Figure 4.24B), even though no staining treatment was performed. Additionally, while the contrast of these fibers was much darker than nonmineralized fibrils, there was no diffraction from any part of the fibril (inset), indicating that the material providing contrast was not crystalline. As indicated earlier, the same thing can be observed for bone, as was shown in Figure 4.13B, which is an isolated collagen fibril from equine bone which has nondiffracting amorphous CaP within the fibrils.

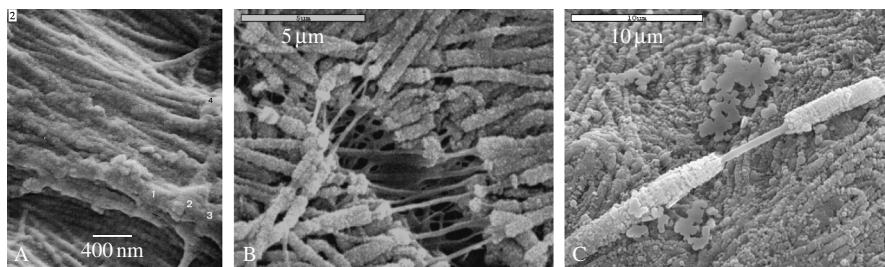


Figure 4.23 Comparison of mineralized collagen sponges to native bone. (A) SEM micrograph of native bone of the surface of whole bone from canine femur diaphysis. (Reproduced by permission of Bagambisa FB, Joos U, Schilli W, A Scanning Electron Microscope Study of the Ultrastructural Organization of Bone-Mineral, *Cells and Materials*, 3(1): 93–102 (1993).) Bundles of collagen fibers are described as being densely invested in mineral, sometimes causing bulging and clubbing of the fibers, and a granular surface texture. The authors noted that some indications of collagen periodicity could be discerned in the loosely packed fibers (see lower left and upper right regions of the image). (B) Cellagen™ sponge mineralized with CaCO₃ PILP phase. (C) Cellagen sponge mineralized with CaP PILP phase. Note the nondescript appearance of the mineral phase in all three cases. The presence of platy intrafibrillar nanocrystals is not at all apparent when examined by SEM, which gives an entirely different impression of the mineral phase than TEM.

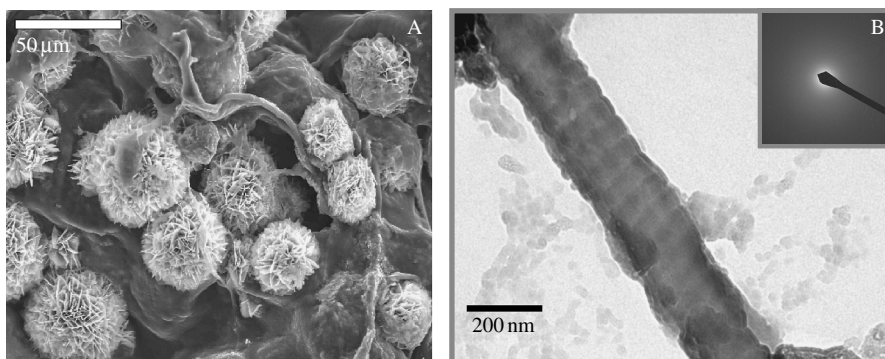


Figure 4.24 Comparison of crystallization mechanisms. (A) The control reaction, which did not use a polymer process directing agent, produced spherulitic clusters of HA on the surface of the fibers, similar to those reported in the literature. (B) With the addition of polyaspartate (15 μg/ml, $M_w = 6200$ Da), the collagen fibrils became infiltrated with an amorphous precursor phase, as evidenced by this unstained fibril, which shows a pronounced banding pattern, but does not diffract (inset).

The amorphous precursor subsequently transformed into the HA phase, as determined by both X-ray (Figure 4.25) and electron diffraction (Figure 4.26). To our surprise, not only were the collagen fibrils embedded with nanocrystals of HA (Figure 4.26A), but the crystals were oriented in the proper [001] direction parallel to the collagen fibrils, as occurs in bone (Figure 4.26B). The nanocrystals

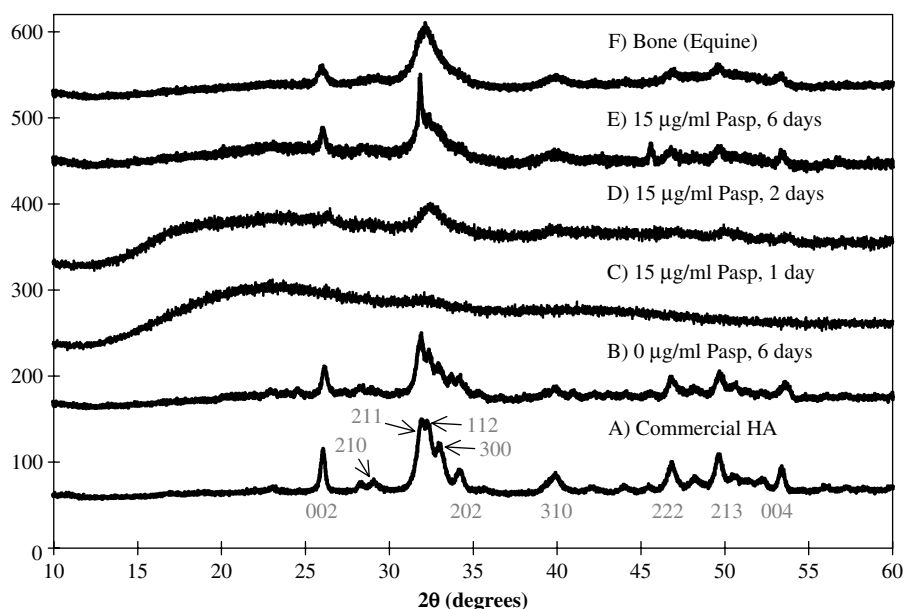


Figure 4.25 X-ray diffraction (XRD) spectra of calcium phosphates crystallized in the absence and presence of polyaspartic acid. The bottom spectrum (A) is of a commercial hydroxyapatite (HA) standard showing the typical diffraction planes present in a randomly oriented powder pattern of HA. Spectrum (B) is of mineralized collagen in the absence of polyaspartic acid, which serves as a control sample that only produces HA clusters on the surface of the sponge (Figure 4.2C). Therefore, the same planes are expressed as the standard HA due to the high degree of crystallinity and random orientation of the crystallites in the clusters. Spectra (C–E) show stages of the PILP mineralization process in which collagen samples were removed from the mineralizing solution at 1, 2, and 6 days respectively. As can be seen, the mineral phase was initially amorphous in the early stages, and gradually transformed into poorly crystalline HA. Note the similarity in spectrum (F), which is of equine bone, to that of the fully mineralized sample (E), both of which exhibit relatively broad peaks, presumably due to the extremely small size of the intrafibrillar crystallites (or crystal lattice strain and/or paracrystallinity).

are not clearly seen with conventional bright field TEM (Figure 4.27A), but using the electron beam diffracted from the (002) spot, dark field TEM nicely illuminates the numerous [001] oriented nanocrystals that are lined up within the fibril (Figure 4.27B). Selected area electron diffraction (SAED) patterns of several isolated fibrils showed the same patterns, which are essentially identical to bone (comparison shown in Figure 4.26). This was not really expected since we had not added any ‘specific’ nucleating proteins. Our only goal was to achieve intrafibrillar mineralization. Instead, the interaction between amorphous precursor and collagen fibrils was apparently sufficient to ‘regulate’ the nucleation event during the amorphous to crystalline transformation.

There was one particular region of a mineralized sample that may be providing clues as to how the controlled uniaxial orientation of HA crystals is achieved. Usually, the crystals cannot be readily discerned when they are embedded in the

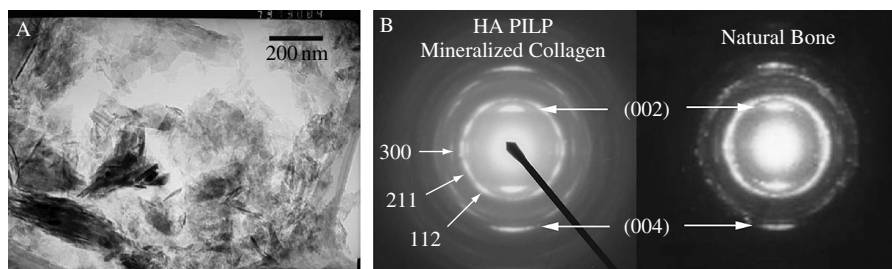


Figure 4.26 Characterization of mineral component of CaP-collagen composites. (A) TEM of crystals extracted from the composites shows nanoscopic crystals with a roughly platy morphology, although it was difficult to separate and isolate the crystals since they were strongly agglomerated, such as in the bundles at the bottom left. (B) Comparison of selected area electron diffraction (SAED) from our CaP mineralized collagen sponge to that of native bone. (Reprinted with permission of John Wiley & Sons from Ziv V, Sabanay I, Arad T, Traub W and Weiner S, Transitional Structures in Lamellar Bone, *Microscopy Research and Technique*, 33(2): 203–213, Copyright © 1996 John Wiley & Sons). The arcing of the (002) and (004) planes indicates that the HA crystals are not perfectly uniaxially oriented along the *c*-axis of the collagen fibril (the angle subtended by the arcs indicates a misorientation of up to 15°). Additionally, three other planes have spots spread into arcs, with *d* spacings that correlate to the (112), (211) and (300) planes. The *d* spacings of these other diffracted planes are extremely close, so a combination of the three arcs imparts the diffraction pattern with what appears to be the characteristic ring observed in bone (the arcs are more separated in our sample, making the separate spacings distinguishable). It is important to realize, however, that this is not really a powder ring, but corresponds to three distinct sets of planes. The simultaneous presence of these other arcs indicates that the crystals also have some rotational disorder, in addition to the tilt disorder. While the latter has been mentioned in the literature, the rotational disorder has not. In fact, the schematics that dominate the literature always indicate that bone consists of parallel aligned platelets, but such illustrations are somewhat misleading since such an organization would not lead to this diffraction pattern.

collagen fibrils (such as the nondescript mineral described earlier), but in this particular region, the edges of crystals can be seen as striations along the fibrils (Figure 4.28A). In addition, whole clusters of crystals appear to ‘escape’ from the constraint of the collagen, leading to what appears to be partial spherulitic bundles (Figure 4.28B). This seems to suggest that the crystals that are formed within collagen are following their normal growth pattern (i.e. nucleating and/or growing in the [001] direction), except that they are forced to lie in a more parallel arrangement by constrained growth within the collagen fibrils. This explanation may not be appealing to some people, who favor the concept of a highly specific nucleating protein, but given the similar features found in the TEM images, and the *identical* electron diffraction data from this model system, this is an alternative view that should be considered. Clearly the most specific nucleating protein that generates the bone like orientation of the HA crystals is the collagen, since polyaspartic acid was the only NCP added to our reaction. It is interesting that this preferred orientation only arises when the crystals are nucleated from an amorphous precursor phase, and not from solution (as in the control reaction).

Another interesting observation is the structure of the crystals that is revealed upon removal of collagen by bleach treatment (Figure 4.29). This sample was

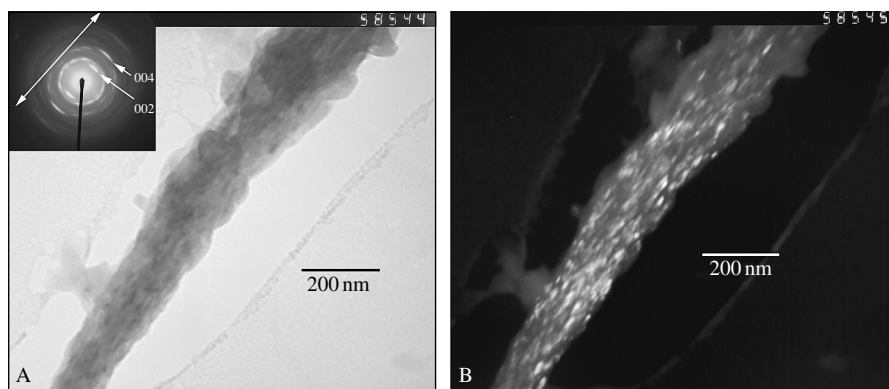


Figure 4.27 Examination of mineralized sponge after crystallization of the amorphous phase shows that the collagen fibers are embedded with nanocrystals of HA. (A) TEM of an isolated fiber shows contrast from mineral phase, but the crystals are not readily apparent. Selected area electron diffraction (SAED) of the fibril (inset) demonstrates that the mineral phase is hydroxyapatite, and that the (002) and (004) planes are oriented parallel to the long axis of the fibril (arrow). The arcing of the diffraction spots, which is the same as in natural bone, indicates that the crystals are tilted with a slight mis-orientation along their *c*-axes. (B) Dark field TEM was performed by illuminating the crystals from the beam of 002 diffraction spot, demonstrating that the fibril is loaded with crystals that are uniaxially oriented in the [001] direction along the long axis of the collagen.

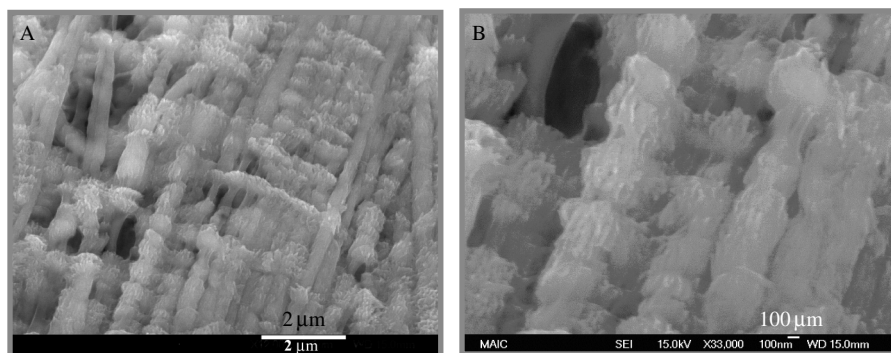


Figure 4.28 Mineralized sample with a region where the embedded crystals protruded from the fibers. (A) The fibrils appear to be loaded with crystals, and the protruding edges of the crystals appear to be roughly uniaxially aligned with the collagen fibrils. (B) In this particular region, the crystals appear to be emerging from the constraints of the collagen fibers, at which point they start to grow outward in the more typical cluster arrangement.

evidently fully mineralized, as can be seen by the remaining mineral which retains the fibrillar shape after removal of the collagen. Figure 4.29B shows the appearance of collagen alone, which is distinctly different than the remnant mineral. Energy dispersive spectroscopy also confirms that these remnant fibrils are composed

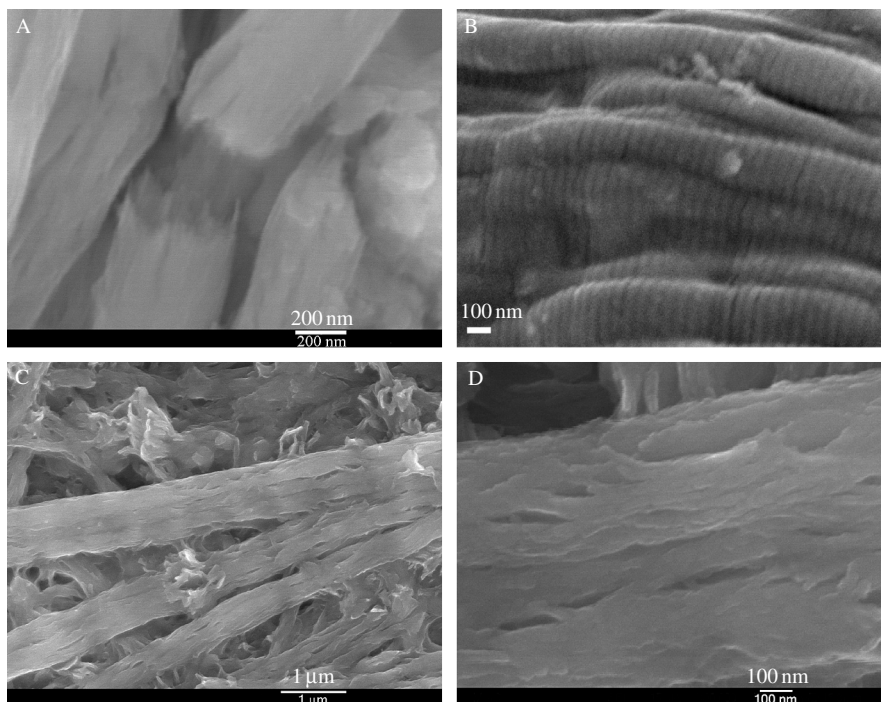


Figure 4.29 Deproteinization of the CaP mineralized collagen composites reveals the mineral architecture within. (A) This fractured fibril shows crystals that span the entire diameter of the fibril, demonstrating full intrafibrillar mineralization. (B) For contrast, this SEM micrograph shows the appearance of collagen alone (prior to mineralization), which is distinctly different than the deproteinated samples. (C) Even after removal of the collagen, the coherent structure of the remaining mineral also indicates a high degree of mineral infiltration occurred within the preexisting fibers. The mineral appears to have a fibroplaty texture, with long crystals that apparently meandered along the interstitial space of the fibers as they were being formed. (D) Although we cannot say for certain that the mineral was not altered by the bleach treatment, these images do fit the diffraction data quite well. The tilt disorder is readily apparent, and there even appears to be some rotational shift as the crystals meander and perhaps twist as they traverse the fibers.

of CaP. The brittle nature of the fractured fibril in Figure 4.29A also has a different appearance than is seen for organic collagen fibrils. Crystallites can be seen to span the diameter of the fracture surface, indicating that full intrafibrillar mineralization was achieved. Unlike the CaCO_3 mineralized collagen, the HA crystals are more distinct, such that the roughly parallel alignment can be easily discerned (Figure 4.29). The crystal shape is somewhat difficult to define, and appears to be platyfibrous as the crystals traverse along the collagen fibril. It is easy to see how there could be both tilt and rotational disorder of the crystals, as is quantitatively identified by the electron diffraction patterns. Of course we cannot be certain that these crystal shapes are the same as in bone, but it is interesting

to consider that such crystals could easily be described as ‘needle-like’ or platy, depending on the extraction and characterization procedure. For example, crystals extracted from these composites do appear to be more platy when examined by TEM (Figure 4.26A). Perhaps this offers an explanation for the different descriptions of bone crystals found in the literature.

Specificity of Mineral

It is interesting to note that our preliminary results with the CaCO_3 system were not well received by the bone research community, because it was considered that CaCO_3 biom mineralization is not relevant to CaP biom mineralization. It is understandable why the CaP researchers would not consider CaCO_3 results relevant, because they are only considering the traditional view of crystal nucleation and growth, where an additive would simply be considered as a nucleation promoter or inhibitor, or growth modulator, for example. If this were the case, then ‘specific’ protein–crystal interactions that occur for CaCO_3 would not be relevant to CaP, since the crystal lattice planes differ. In other words, the specific change in morphology of a crystal growth modifier would not be manifest in another entirely different crystal structure. It is for this reason that we have spent so much effort here emphasizing this newly defined role of ‘nonspecific’ acidic macromolecules as being process directing agents. As we have tried to show, even though the final product may differ, there are certain aspects of biom mineralization that can be understood from examination of crystallization mechanisms at a fundamental level.

The PILP process *does* appear to be applicable to a variety of mineral systems (CaCO_3 , CaP, CaOx, BaCO_3 , SrCO_3), and therefore fundamental aspects of the process, such as the capability of molding crystal shapes, may be relevant across different mineral systems. We should point out though, that even though we have preliminary evidence to suggest that a fluidic precursor can be synthesized in the different minerals, the resulting crystal products do differ. In fact, with the CaP system, when the amorphous precursor phase transforms, the driving force for forming very small crystals leads to a highly polycrystalline film of platelets, unlike the CaCO_3 system, where large single crystals could be molded when they retained the shape of the precursor.

Differences were observed in collagen mineralized by the two different mineral systems, but rather than ignoring the results because of this difference, we believe useful information about bone formation can be gleaned from such comparisons. For example, the deproteinated samples exhibited a very different mineral texture. The CaP composites seem to be more uniformly infiltrated with crystal. Another example of the CaCO_3 mineralized collagen shown in Figure 4.30 shows a strikingly different texture. Selected area electron diffraction exhibits a single-crystalline spot pattern for this mineralized fiber (Figure 4.30B). The striations suggest the crystals are fairly large and blocky (note, this was not true of most samples examined). It is not clear how the crystals grew to such a large size given the dimensions of the gaps and grooves in collagen. In fact, this same puzzle is found for bone crystals, which

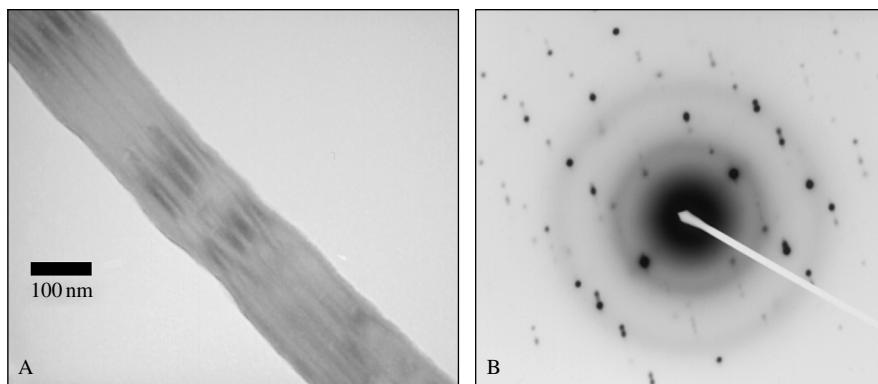


Figure 4.30 Unusual specimen of CaCO_3 mineralized collagen. (A) The striations in this fibril are likely large aligned crystals that yielded the selected area electron diffraction pattern in (B). The spot pattern indicates that the composite is either composed of single-crystalline calcite, or many nearly perfectly aligned crystals. There are doublet spots from some crystals that are slightly misoriented, but the texture is much more aligned than those seen for the CaP composites.

seem to outgrow the nanoscopic space within the fibrils. Therefore, we surmise that the organic matrix, which should be quite flexible in the swollen hydrated state, must be pushed aside as the crystals are formed (or when the matrix is infiltrated with precursor phase).

The fine scale morphology of HA might be an indication as to why this mineral was evolutionarily selected for vertebrate hard tissues, as opposed to CaCO_3 , which is the main mineral phase observed in invertebrates. Calcite forms very large crystals relative to hydroxyapatite (due to their different solubility products and surface energies), and although we have shown that intrafibrillar mineralization can occur with CaCO_3 , this tendency to form larger crystals could be less optimal. Structurally, this would not be as beneficial because it would allow crack propagation to occur rapidly through the blocky solid regions. The very fine scale texture of the embedded hydroxyapatite phase provides a large interfacial area across the brittle–ductile interface.

4.2.8 CONCLUDING REMARKS ON BONE FORMATION

Although our results are based on an *in vitro* model system, they appear very promising thus far. The data fully supports our hypotheses, but more work needs to be done to understand the mechanism leading to intrafibrillar mineralization. At this point, we can definitively claim that the mineralization process occurs through a precursor phase (which is either amorphous or paracrystalline), we do not have proof that the precursor infiltrates the collagen fibrils via capillary action. More importantly, formal proof that this PILP process occurs in the natural process of bone formation still awaits direct *in vivo* evidence, as is true of most biomineralization theories.

4.3 KIDNEY STONES

Kidney stones are complex biocomposites composed of inorganic crystals and organic matrix. The inorganic part, mainly calcium oxalate and calcium phosphate [202,203], comprises the bulk of the material in the most common types of stones (Figure 4.31). The organic matrix makes up 2–3 % of the total dry weight of stones [204]. Calcium oxalate, CaC_2O_4 , is the most commonly observed inorganic mineral in kidney stones [202,203]. Among the crystallographic forms of calcium oxalate, calcium oxalate monohydrate (COM) and calcium oxalate dihydrate (COD) are the more thermodynamically stable phases, and thus are more prevalent in stones than the unstable calcium oxalate trihydrate (COT) [205]. The second most abundant mineral in stones is CaP, commonly in the structure of hydroxyapatite (HA), with a (simplified) molecular formula of $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ [203]. For comparison to the morphology of crystals found in stones, the inorganic habits of HA, COM and COD are shown in Figure 4.3. Other polymorphs of CaP in stones include carbonated apatite, brushite and OCP [203]. In this review, the roles of the inorganic minerals and their associated organic matrix are discussed with respect to the mechanism(s) of kidney stone formation from a materials chemistry perspective (i.e. we do not consider the physiology of disease states, biological transport of ions across cell membranes, etc.). First we will present a brief summary of some of the prevalent theories in the literature on stone formation, which will provide the basis for presentation of our own hypotheses.

4.3.1 FREE VS FIXED PARTICLE MECHANISMS

Supersaturation of urine with mineral ions is a common phenomenon for both healthy and stone forming patients [206,207]. This means that the precipitation of crystals within the urinary tract can occur; but the formation of crystals is not necessarily problematic as long as the particles are not large enough to become lodged in the tubules and cause discomfort or blockage. It has been proposed that kidney stones can be produced through either a free or fixed particle mechanism [208,209]. The free particle mechanism is considered to arise if a kidney stone is formed from nucleation and growth of free standing crystals in the urinary solution. This mechanism has been questioned because earlier calculations show that the rate of growth of a single particle is slower than the transit time of the crystal within the kidney, which is about several minutes [208]. Thus, the probability of forming a large kidney stone via growth of a single crystal is extremely small. However, the free particle mechanism has been recalculated using new data on nephron dimensions, supersaturation and crystal growth rates, and simulations for acute hyperoxaluria show that precipitated single crystals in solution may have sufficient time to grow and aggregate to large enough dimensions to form a stone [209].

The low probability of forming kidney stones through the free particle mechanism has led to the adoption of the fixed particle mechanism. In this scenario, crystals

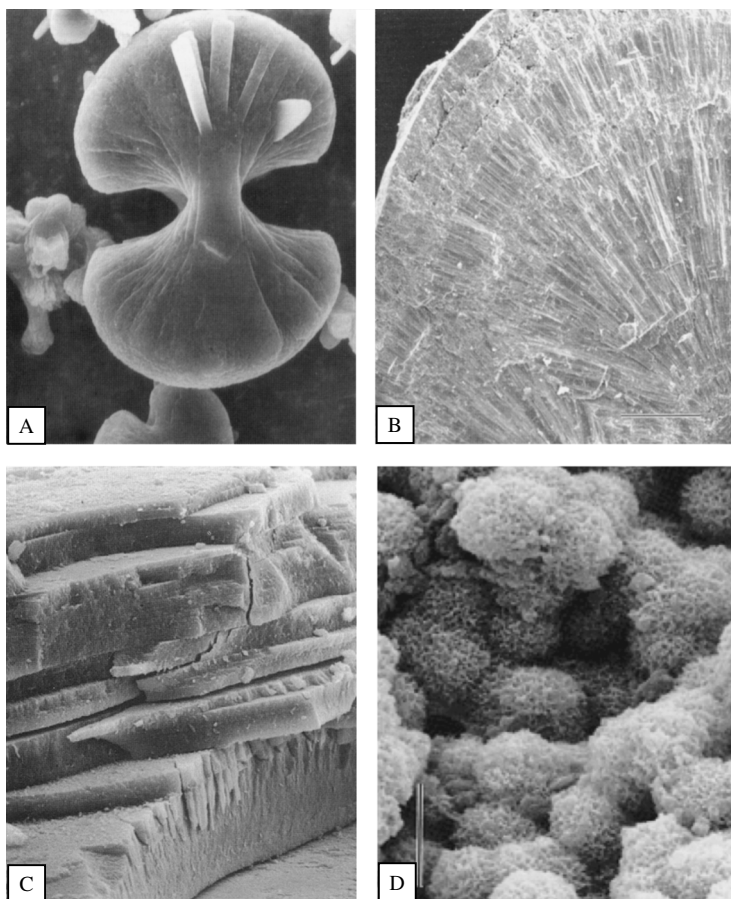


Figure 4.31 Examples taken from the literature of crystals found in kidney stones. Although these stones differ in composition and appearance, the polycrystalline radial growth pattern seems to be a common factor. Note, these examples emphasize spherulitic morphologies, but there are a variety of crystal morphologies found in stones that are not represented here. (A) The dumbbell morphology is commonly seen for crystals extracted from urine. As judging from the platy habit of the individual polycrystals, these are likely composed of COM. (Reproduced by permission of Springer-Verlag from Khan, SR, Hackett, RL, Crystal-Matrix Relationships in Experimentally Induced Urinary Calcium Oxalate Monohydrate Crystals, an Ultrastructural Study, *Calcified Tissue International*, 41: 157–163 (1987).) (B) This stone has a spherulitic texture, composed of densely packed polycrystals that radiate from a central core. Concentric laminations are common in these types of stones. (Reproduced by permission of CRC Press from Khan, SR, Structure and Development of Calcific Urinary Stones in *Calcification in Biological Systems* (ed. Bonucci E), 345–363, CRC Press, Boca Raton (1992).) In (C), it is not clear if there is radial growth, except for the striations on the bottom layer which seem to suggest this is the case. This stone, which exhibits a pronounced laminated growth, is composed of calcium oxalate. (D) An agglomeration of spherulitic clusters of calcium phosphate. HA commonly forms clusters such as these when nucleated on organic substrates. (Reproduced by permission of CRC Press from Khan, SR, Structure and Development of Calcific Urinary Stones in *Calcification in Biological Systems* (ed. Bonucci E), 345–363, CRC Press, Boca Raton (1992)).

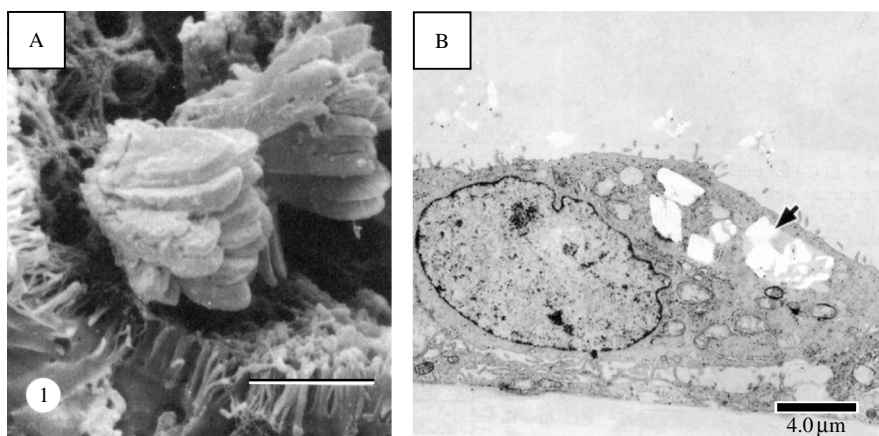


Figure 4.32 Crystal interactions with renal epithelial cells. (A) Aggregates of COM crystals attached to the wall of the proximal tubule in a rat nephron. Bar is 5 μm . (Reproduced by permission of Khan, SR, Hackett, RL, Retention of Calcium-Oxalate Crystals in Renal Tubules, *Scanning Microscopy*, 5: 707–712 (1991).) (B) Transmission electron micrograph of a monolayer of renal epithelial cell lines (Madin–Darby canine kidney, MDCK) that is exposed to oxalate. Arrow indicates ghosts of calcium oxalate that are endocytosed. (Reproduced by permission of Lippincott, Williams and Wilkins from Khan SR, Byer KJ, Thamilselvan S, Hackett RL, McCormack WT, Benson NA, Vaughn KL, Erdos GW, Crystal-cell Interaction and Apoptosis in Oxalate-associated Injury of Renal Epithelial Cells, *Journal of the American Society of Nephrology*, 10: S457–S463 (1999).)

become attached to the renal tubules (Figure 4.32A) [210,211], and the attachment causes a cascade of cellular events leading to an increase in organic matrix which may be deposited onto the crystals, thus stimulating further growth or aggregation and enlargement of the forming stone [212–214].

Crystal–Cell Interaction

Crystal retention by epithelial cells has been highlighted in the fixed particle mechanism. The cells present membrane surfaces where crystals can nucleate or attach, which then provide the time factor required to form into a large kidney stone. Several epithelial cell lines from animals [215–217] have been used to study interactions between cells and crystals. Crystal attachment has been observed to change the physiology of renal epithelial cells [212–214]. Attached calcium oxalate and hydroxyapatite crystals have been endocytosed by cultured renal epithelial cells *in vitro* from monkey and canine cell lines (Figure 4.32B) [215,218]. Endocytosis of urinary crystals in humans has also been observed following renal biopsy of patients with hyperoxaluria [219,220]. An increase in the number of cells has also been reported subsequent to crystal attachment [215]. This translates into possible cell detachment from the basement membrane during cell proliferation, and release

of cells or cell fragments into the urine. This increases the surfaces that can promote heterogeneous crystal nucleation.

Other studies have shown that crystals, e.g. CaOx [221,222] and brushite [223], can cause cellular injury upon attachment. Also, increased levels of oxalate in the urinary tract can cause cellular damage [210,212,216]. Cell injury and/or death may cause reorganization of the membranes such that acidic phospholipids are exposed on the cell surface, and such acidic lipids are probable promoters of calcific stone nucleation or attachment [224–226].

4.3.2 THE COMPLEX URINARY ENVIRONMENT

Regardless of whether one considers the initiation of a stone as occurring in solution or on a cell surface, the formation of calculi in the renal tract is brought about by the decrease in free energy of the supersaturated solution associated with the formation of a new phase (Figure 4.4). Urine contains a large amount of organic matter which can provide surfaces to promote heterogeneous nucleation within the solution [227]. Preexisting inorganic particles in urine can also lower the surface energy and promote nucleation [228–230]. Although heterogeneous nucleation is the likely route of stone initiation in a metastable solution (either on particles floating within the urine or on the cell surface), homogeneous solution grown precipitates should not be discounted, because *in vitro* studies with CaP have demonstrated nucleation from solution and eventual attachment of the precipitates to self assembled monolayer surfaces [231]. This could be considered analogous to the situation where precipitates in urine could adsorb onto the membranes of renal epithelial cells. Once adsorbed, the continued accretion of organic and inorganic material could lead to a stone. This concept is supported by the fact that the nidus of stones, which can often be identified in scanning electron microscopy (SEM), are not always located at the outermost edge, where one would expect if the stone initiated at the cell membrane. On the other hand, the nidus is not usually located in the exact center of the stone either (Figure 4.33), indicating that interaction with the cell surface at some early stage is likely a significant factor in the retention of the stone for subsequent growth [232]. In either case, it is clear that the organic matrix plays a significant role in the formation of a stone.

Organic Constituents

Composition of the Organic Matrix

The organic matrix extracted from urine and kidney stones has been determined to be composed of proteins, lipids and carbohydrates [233–235]. These materials may come from the usual constituents found in urine and blood, as well as detached renal epithelial cells and cellular degradation products [227,236]. Not all organic matrix is believed to be important in kidney stone formation. Fractions of the organic matrix

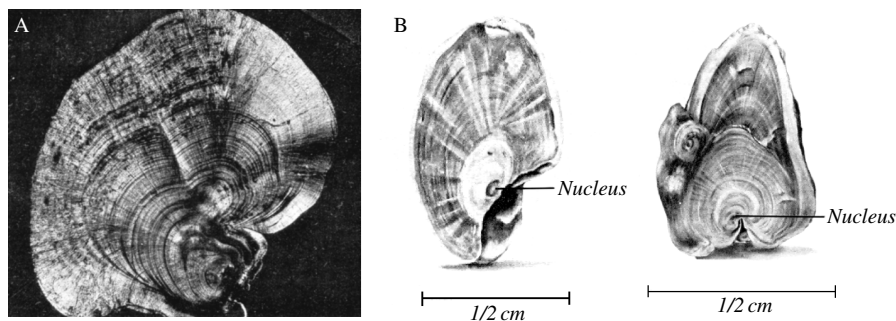


Figure 4.33 Example of stones with an off center nidus. Photomicrograph of the cross section of a calcium oxalate stone showing an off center nucleus. (Reproduced by permission of Springer-Verlag from Pyrah, L. N. in *Renal Calculus*, Springer-Verlag, New York (1979).)

are said to be adventitious, in which the material appears to be adsorbed from the urine onto the forming stone, but without significantly modifying the mechanism of stone formation [237]. Other organics have been found to have strong interactions with the inorganic materials, suggesting that they could modify how calcium oxalate and calcium phosphate nucleate, grow and aggregate into stones.

In the earlier studies of kidney stones, the organic matrix was mainly studied through collection of urinary stones [204]. Recently, a method has been adopted to pinpoint the important components of the organic matrix [238,239]. In this procedure, precipitates are induced from collected urine through addition of ions found in the inorganic component, e.g. Ca^{2+} and $\text{C}_2\text{O}_4^{2-}$. The organic matrix that is assimilated within the precipitated crystals is considered likely to be involved in stone formation (Figure 4.34). The significant proteins, lipids and carbohydrates are then evaluated through extraction of the organic matrix from the induced crystals.

(1) *Proteins*

Proteins have been analyzed in the urine of both normal patients and stone formers, revealing that the same types of proteins are prevalent in both urine samples [240]. Proteins, e.g. Tamm–Horsfall protein, albumin and osteopontin, have been extracted at high amounts in the urine [240]. Meanwhile, another protein, urinary prothrombin has also been identified, but in small amounts in the urine [240]. During induction of calcium phosphate and calcium oxalate in collected urine, different levels of proteins association within the crystals are observed. Tamm–Horsfall protein is partially excluded from the precipitated calcium oxalate [240,241]. Urinary prothrombin, which is observed in small quantities in urine, has been extracted from matrices of calcific stones at considerably higher levels [242]. This suggests that protein incorporation into these precipitated CaOx and CaP minerals may be a highly selective process [241]. This is not surprising given the variability in functionality of the organics, which would be expected to experience different affinities for inorganic particles of variable surface potentials (and particularly ionic crystals). In fact, the adsorption characteristics could change as the particle traverses the renal

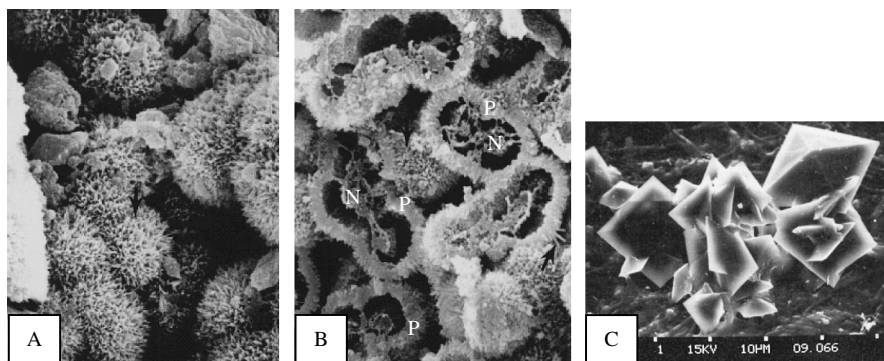


Figure 4.34 Crystal formation induced from urine. (A) Scanning electron micrographs of induced CaP (with some CaOx) stones from urine. (B) EDTA demineralized CaP crystals leaving behind EDTA-insoluble organic matrix. (Figures 4.34A and B are reproduced with kind permission of Springer Science and Business Media from Khan SR, Atmani F, Glenton P, Hou ZC, Talham DR, Khurshid M, Lipids and Membranes in the Organic Matrix of Urinary Calcific Crystals and Stones, *Calcified Tissue International*, 59: 357–365 (1996).) (C) Aggregates of COD crystals typically formed in urine samples of stone formers ($\times 2000$). (Reprinted from Rodgers AL, Spector M, *Crystallographic Analysis of Urinary Calculi* (ed. Rous SN), 47–55, Grune and Stratton, Inc., Orlando, Copyright 1987 with permission from Elsevier.)

tract since the pH changes throughout, which in turn affects the surface potential of the particles and organics.

The capacity of calcium oxalate and calcium phosphate to accommodate proteins differs as well. Osteopontin has been found to be incorporated into calcium oxalate monohydrate and hydroxyapatite but not brushite [240]. However, more urinary prothrombin is extracted from calcium oxalate monohydrate crystals than in calcium phosphate or calcium oxalate dihydrate [243]. Tamm–Horsfall protein is more likely to bind with calcium phosphate crystals than with calcium oxalate [243].

Once the proteins get adsorbed onto the crystal surface, the proteins may serve to promote further crystal growth, as suggested by *in vitro* studies of immobilized proteins, which have been shown to promote nucleation of crystals [244,245]. On the other hand, some incorporated proteins are thought to be growth inhibitors. Urinary prothrombin [246,247], osteopontin [248,249], and nephrocalcin [250–252], have been observed to inhibit crystal growth *in vitro*. Proteins can also act as aggregation promoters or inhibitors. For example, osteopontin has been shown to be a potent inhibitor of aggregation [249], while Tamm–Horsfall protein can reportedly inhibit or promote aggregation, depending on the experimental conditions (e.g. pH and ionic strength) [253]. Given the likelihood of cross interactions between the multitude of variables in the complex urinary environment, this is one area that can benefit from using experimental design techniques to help sort out conflicting results [254,255]. The adsorption of an organic to a particle can change its surface potential (which will depend on pH and ionic strength), which in turn can influence whether the particle–particle interactions are attractive or repulsive.

Adsorption of large macromolecules can even create a steric barrier which prevents particle aggregation [76]. Aggregation studies have nicely benefited from the recent development of colloidal probe techniques with the AFM, which can be used to experimentally measure the interaction forces between a crystal attached to the tip of the AFM cantilever with various surfaces [256–261] (such as another crystal or a mimetic lipid membrane), and in the presence of additives in the solution. There are many proteins in urine which we cannot describe here; and instead refer the reader to the useful review article provided by Khan and Kok [262].

(2) Lipids

Different types of lipids are found in urine. Neutral lipids (e.g. cholesterol and cholesterol esters) and glycolipids (e.g. D-sphingosine and cerebrosides) are present in higher quantities compared to phospholipids [235]. Generally, lipids in urine of stone formers are in higher amounts compared to that of nonstone formers [226,263]. This increase in the amount of lipids may have been caused by the change in physiology of the epithelial cells brought about by oxaluria (high oxalate levels) or crystal attachment, both of which can cause cell damage [213,218,264].

Similar to protein assimilation, the incorporation of lipids is a selective process [235]. Although there are a number of neutral lipids and glycolipids in urine, only a few of these get assimilated into the induced CaOx and CaP crystals. Meanwhile, the small amounts of phospholipids found in urine are almost all assimilated into the inorganic material. The seemingly biased incorporation of phospholipids into the crystals has led to the study of the role of phospholipids in crystallization. Methods employing extracted lipids from urinary stones [265], renal epithelial cells [266] and synthetic phospholipids [267–269], have been used to analyze phospholipid and calcific mineral interaction. Langmuir monolayers have been useful for *in vitro* studies on the nucleation behavior of COM on mimetic phospholipid monolayers (Figure 4.35A). Crystals with the ($\bar{1}01$) face parallel to the monolayer are amply formed on negatively charged phospholipids, e.g. phosphatidylglycerol and phosphatidylserine [268–270], presumably because the ($\bar{1}01$) face exposes a calcium rich plane that interacts with the negative charge on the acidic phospholipids (Figure 4.35B) [204,271,272].

The Langmuir monolayer studies have also shown that the mobility of these lipid molecules is important as well [226,273]. Less rigid phospholipid monolayers are proposed to localize charge density that can induce crystal nucleation and attachment. In cells, this localization of charge density may be due to lateral and transmembrane movement brought about by oxaluria or crystal attachment. Phosphatidylserine, which is found in the inner leaflet of the plasma membrane, inverts and becomes exposed to the cell surface during injury or apoptosis, and thus could promote calcific stone formation [224,266,274]. Lipid reorganization may also lead to phase segregation within the membrane. *In vitro* studies of biphasic (e.g. liquid expanded–liquid condensed) phospholipid monolayers produced COM crystals at the boundaries of the phases (Figure 4.35C). There is strong evidence, based on the promotion of calcific crystallization on renal tubule cells, as well

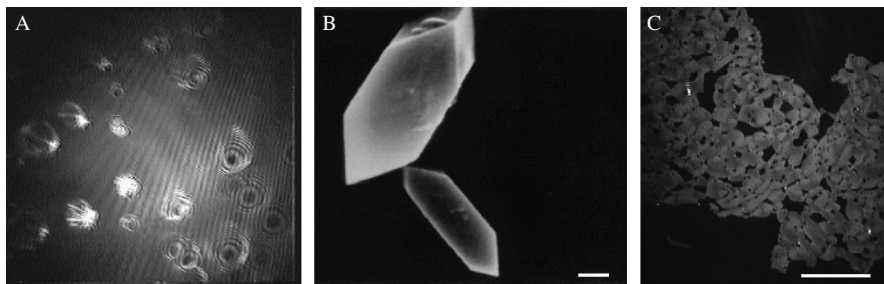


Figure 4.35 CaOx crystals grown under Langmuir monolayers. (A) Langmuir monolayer of a compressed phospholipid (dipalmitoylphosphatidylglycerol) viewed using Brewster angle microscopy. The bright spots are COM crystals that have nucleated under the monolayer. Image is 2 mm $\times$ 2 mm. (B) SEM micrograph of COM crystals grown under a DPPC phospholipid monolayer. The crystals were oriented with their (10 $\bar{1}$) face toward the monolayer. Bar = 1 μ m. (Figures 4.35A and B are reprinted from *Journal of Crystal Growth*, Vol. 192, Whippis S, Khan SR, O'Palko FJ, Backov R, Talham DR, Growth of Calcium Oxalate Monohydrate at Phospholipid Langmuir Monolayers, 243–249, Copyright 1998, with permission from Elsevier.) (C) Brewster angle micrograph of a compressed phospholipid (dipalmitoylphosphocholine) exhibiting two phases: liquid expanded (dark background) and liquid condensed (light gray). Calcium oxalate crystals, which appear as bright spots, are observed at the interface between the two phases. Bar = 100 μ m. (Reprinted with permission from Benitez IO, Talham DR: Brewster Angle Microscopy of Calcium Oxalate Monohydrate Precipitation at Phospholipid Monolayer Phase Boundaries, *Langmuir*, 20: 8287–8293, Copyright 2004 American Chemical Society.)

as *in vitro* phospholipid models, to support the hypothesis that lipids and cellular membranes are important in the genesis of kidney stones.

(3) Carbohydrates

Glycosaminoglycans (GAGs), a group of long chain carbohydrates, have been extracted from kidney stones, and constitute approximately one third of the organic matrix [204,234]. Heparin sulfate and hyaluronic acid are the most abundant GAGs extracted from calcium oxalate stones [234,275,276]. Although chondroitin sulfate is common in urine, this macromolecule is excluded from incorporation into calcium oxalate stones [275]. The selective process of incorporation of GAGs is also observed [277], similar to that found in proteins and lipids. The complexity of such large macromolecules makes them difficult to study; but at the same time, their large size suggests that these species could be particularly important in stone formation, because inhibitory potential (in crystal nucleation/growth/aggregation) is often strongly enhanced with an increase in size of the macromolecule. The highly acidic nature of these macromolecules could also contribute to the formation of amorphous phases (we have not yet examined these systems).

Selective Incorporation of Organic Matrix

Only a portion of the organics in urine gets included in kidney stones. These are observed when calcium oxalate and calcium phosphate crystals are precipitated out

of the collected urine of stone and nonstone formers. The preferential incorporation of the organic matrix has been correlated to its interaction to the crystallographic planes of the crystals [251,278,279]. This process has been observed for proteins, lipids and carbohydrates as discussed in the previous sections. The selectivity of organic matrix within the kidney stone has been proposed to be an indication of a functional use of the organic matrix to help inhibit stone formation, which is somehow not as effective in stone formers [280].

Recently *in situ* atomic force microscopy of carboxylic acid rich molecules found in urine (e.g. citrate and osteopontin), has been utilized to study the specific interaction of the organic matrix on calcium oxalate monohydrate [281]. Although both molecules are strong inhibitors of calcium oxalate growth [248,249,282], their effects on the COM crystal are seen to arise from interactions with different crystallographic planes. Citrate has an effect on the ($\bar{1}01$) face, while OPN has effect on the (010) face, but not on the ($\bar{1}01$) face [278,281]. Nephrocalcin, another macromolecule found in urine, also specifically interacts with ($\bar{1}01$) face of COM [279]. Fluorescently labeled proteins have also been utilized during the formation of calcium oxalate to validate that certain molecules do preferentially interact at specific regions within the crystal [283]. The biased assimilation of the organic matrix may relate to how the organic matrix is distributed within the kidney stones as they are being formed.

The organic matrix only accounts for a small portion of the total weight in kidney stones, although it can occupy a large volume as it is dispersed throughout the stone [204]. Electron microscopy of the ultrastructure of polycrystalline, as well as single-crystalline, urinary and induced calcific stones, have revealed a considerable amount of organic matter at the core of the material (Figure 4.34B) [233,284,285]. The presence of organic matrix at the core suggests that the organic matrix plays an important role in the initial stages of crystal formation [277,235,286]. In addition, organic matrix is found at the surface of each of the individual crystals making up the stone, which hints of a possible role in aggregation of the crystals.

Organic matrix in polycrystalline stones often parallels the radial striations and concentric laminations commonly observed in stones (Figures 4.31B, 4.31C and 4.33) [233,284,285]. The absence of randomness in the incorporation of organic matrix in calcific stones has led to the suggestion of an ordered ultrastructure [286,287]. We would argue that this so called ‘ordering’ likely arises from the preferential exclusion of impurities as the crystals are trying to form, such as along the radial striations or concentric laminations of a forming spherulite (see discussion in the section on *Amorphous Precursors to Spherulites*).

Inorganic Constituents

Crystallization of only one type of inorganic species is uncommon in kidney stones. Most often, kidney stones are found to have mixtures of CaOx and CaP [203,288]. The relationship between calcium phosphate and calcium oxalate is of clinical importance because recurrent calcific stone formers contain larger amounts

of calcium phosphate than one-time stone formers [289]. Analysis of large calcium oxalate stones often shows a central core of calcium phosphate in the form of apatite (Figure 4.36) [203,232,290]. The common occurrence of a CaP core and CaOx periphery has led to the idea of a possible geometrical relationship between the two crystal phases. Since HA is the most common core, epitaxial growth of CaOx onto this form of CaP has often been suggested as a mechanism contributing to the formation of such mixed composition kidney stones [229]. The structural relationship between different crystallographic planes of these two inorganic species has been calculated [228], however, such correspondence is rarely observed in kidney stones. Although the proper epitaxial relationship has yet to be established between the crystal species, hydroxyapatite has been found to be a good substrate for calcium oxalate nucleation [229,291]. It is important to realize that the presence of any particle or foreign substance in a metastable solution (i.e. the supersaturation is not high enough to surmount the energy barrier required for homogeneous nucleation) can stimulate heterogeneous nucleation by lowering of this energy barrier since the creation of a new surface is not required (e.g. dust works well, as shown in Figure 4.3A). This could include protein aggregates or lipid micelles that

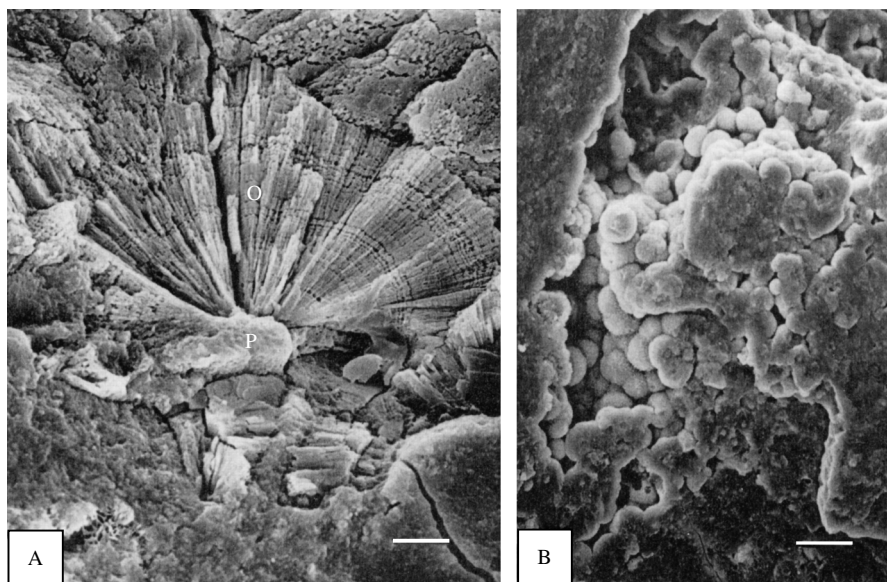


Figure 4.36 Scanning electron micrographs of a human urinary stone with a central core structure. (A) Calcium oxalate crystals, O, exhibit a radial arrangement that emanates from a core structure, P. Energy dispersive X-ray analysis of the core reveals calcium and phosphate peaks. Bar is 5 μm . (B) Higher magnification of the core structure shows rounded concretions, which suggests that the CaP structures are either amorphous or spherulites. Bar is 1 μm . (Figures 4.36A and B are reproduced by permission of Lippincott, Williams and Wilkins from Khan SR, Calcium Phosphate Calcium Oxalate Crystal Association in Urinary Stones: Implications for Heterogeneous Nucleation of Calcium Oxalate, *Journal of Urology*, 157: 376–383 (1997).)

accumulate from the breakdown and release of membrane components of the renal epithelial cells, or from inorganic precipitates such as CaP formed in the earlier segments of the nephron. In addition, given the large amount of organic material within urine, it is likely that much of this material will rapidly adsorb onto and cover the forming crystal surface (to lower the interfacial energy), and so the issue of a highly specific epitaxial relationship between HA and CaOx seems irrelevant if a free HA surface is not available. Such considerations likely arose from the biomineralization literature dealing with biologically controlled mineralization, in which epitaxial interactions may be at play for directing crystallographic phase and orientation in these more controlled precipitations.

Spherulites

Based upon observations from our *in vitro* model system, we believe that the central core in mixed stones may be generated by an amorphous CaP phase, which subsequently transforms into a crystalline spherulitic core. As shown in Figure 4.36B, the central core is composed of rounded concretions that are nonfaceted and may be amorphous. However, one cannot assume that mineral with a spherical shape is amorphous. In other cases, examination of the interior of such spherical objects often shows they are composed of radially oriented polycrystals which emanate from the center of the sphere. This can be seen for the outer CaOx portion of the stone in Figure 4.36A, as well the stone examples in Figures 4.31B and 4.33. This type of crystal structure is called a spherulite. Spherulites are a common crystallographic form, which are not only found in mineral salts, but occur in a variety of materials. For example, organic polymers (the constituents of plastics) commonly crystallize from the melt into spherulites, as do graphitic clusters in cast iron, and electrodeposited metal films. The reason for the similar structures from such dissimilar materials lies in the formation mechanism, which arises from nonclassical nucleation. Spherulites usually form under rapid growth conditions, such as high supersaturation in the case of minerals, or rapid cooling of the melt in polymers (which are long chain molecules that cannot easily organize themselves into periodic crystalline arrays). Such conditions are ‘quenched’ beyond the classical nucleation regime, and kinetic effects dominate the phase transition. The ‘freezing in’ of local orientation fluctuations at the growth front can create new grains, sometimes referred to as secondary nucleation [292].

Spherulites are properly defined as polycrystalline aggregates composed of radial, or sometimes concentric, polycrystals (often illustrated with a child’s toy called a Koosh™ ball). Spherulitic structures are often seen in kidney stones (Figure 4.31), yet spherulitic growth has received little attention from this community [293,294]. Given that this appears to be the central initiating component of many stones, we believe this mechanism of crystallization should be examined more fully. There are several possible scenarios of how this might occur in the renal tract, but before making any suggestions, the semantics of crystal structures needs to be clarified. In particular, spherulites are often described simply as polycrystalline aggregates.

While this is true, they are polycrystalline, this terminology ‘aggregate’ can be very misleading because they are not formed by aggregation of preformed crystals. Instead, spherulites form by a nucleation and growth phenomenon (Figure 4.37). For example, if multiple crystals nucleate from a central core, their growth becomes spatially constrained such that they can only grow outward; or multiple branching (such as from polytwinning or quenching of orientational defects) forces the crystals into a radial growth pattern. Early stage spherulites often exhibit a sheaf of wheat or dumbbell type morphology (where branching occurs at the ends of the central sheaf), while later stages take on the more symmetrical spherical shape (Figure 4.37A or B).

The spherulitic crystal texture can be readily identified with polarized light microscopy if the material is doubly refracting (exhibits birefringence), because a Maltese cross pattern is seen under crossed polars. We find polarized light microscopy to be extremely valuable for examining crystallization reactions. A schematic illustrating the different birefringence patterns of single crystals vs spherulites is provided in Figure 4.38. As can be seen in many of the figures in this review (those with magenta backgrounds), we frequently use the gypsum accessory plate because it is useful for examining crystallizations that proceed through amorphous precursors because it allows one to simultaneously observe both amorphous and crystalline materials (Figure 4.38B). In the case of spherulites, the dark lines of the Maltese cross, which are aligned with respect to the polarizer and analyzer of the microscope, do not change in position as the sample is rotated

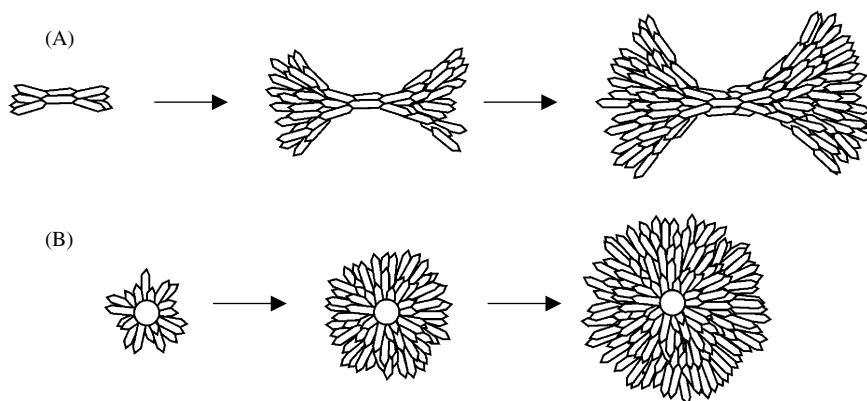


Figure 4.37 Schematic representation of spherulitic growth patterns. (A) Branching occurs during crystal formation, which can arise from a multitude of reasons, such as polytwinning, secondary nucleation or blockage of the growth front from impurities. This can result in partial spherulites, as shown here, which are sometime referred to as ‘sheaf of wheat’ or ‘dumbbell’ morphologies (middle and right image respectively). Alternatively, excessive branching can wrap around to fill the space around the axis, creating a full sphere, or a double lobed sphere if the branching only wraps back partially along the axis. (B) The round spherulite can also result from crystals that grow isotropically from a central core, leading to a more symmetric radial growth pattern. This is illustrated in cross sectional view (as though looking at the fracture surface of a sphere).

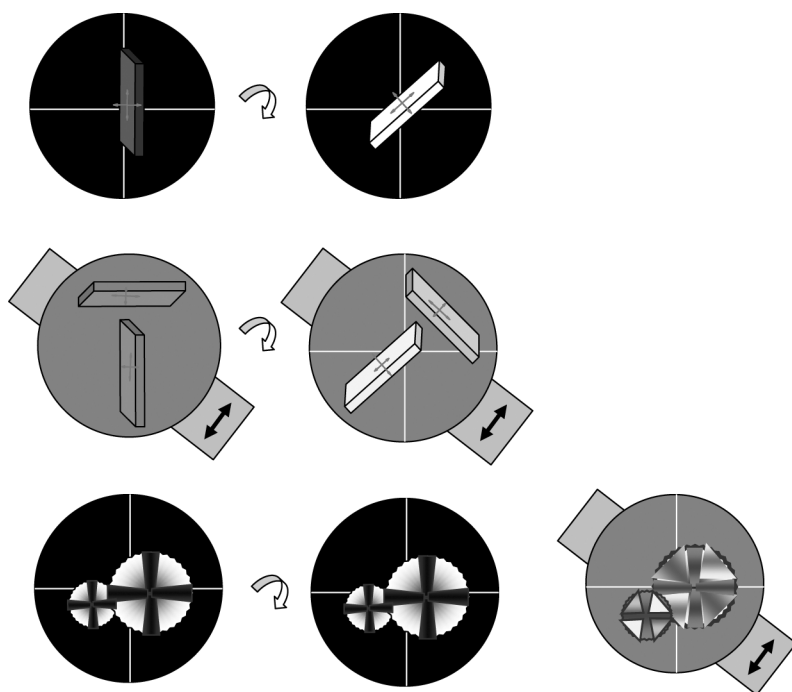


Figure 4.38 (Plate 6) Birefringence patterns of crystals with polarized light microscopy. (A) A biaxial single crystal will appear extinct when the vibration directions of the crystal are parallel to the crosshairs on the microscope, which are set to be parallel to the crossed polars. When the stage (and crystal) is rotated 45° , the crystal will become fully birefringent (brightly illuminated). (B) Retardation plates can be used to identify the optical properties of a crystal, such as slow and fast directions (which appear yellow and blue in this example). We use the gypsum 1st order red λ plate because it enables one to see both amorphous and crystalline materials. The gypsum plate produces an additional 550 nm of retardation, which changes the interference color of white light into magenta. Amorphous materials, or birefringent materials in the extinct position, will appear the same magenta color as the isotropic background. The outline of an amorphous material, which would be dark without the gypsum plate, can then be seen. (C) Spherulitic crystals display a Maltese cross pattern under crossed polars due to the radial orientation of the polycrystals. The Maltese cross remains stationary, even as the sample stage is rotated. When using the gypsum plate, the Maltese cross still arises from the extinct position of the radial crystals, but it will now appear magenta. In addition, the alternate sectors of the sphere will have opposite vibration directions from the opposing direction of the radial crystals, and therefore will display opposing retardation colors.

on the stage (Figure 4.38C). This is because, as the sample is rotated, the radial crystals rotate and put new crystallites into parallel alignment with the polars. When a crystal is aligned with its optic axis parallel to the polar, it will appear dark (the birefringence is extinct). This extinction pattern is distinctly different from the extinction pattern of a single crystalline material (which goes all bright and then all dark as the sample is rotated every 45°). Likewise, a random aggregation of

crystals will just show a random assortment of extinction directions of the individual crystallites. It should be noted that, if the polycrystalline growth is constrained to two dimensions, such as in a film (e.g. Figures 4.8C, 4.42B, 4.43A and 4.49C), or it is restricted to only a partial sphere (from aggregation), the radial texture is still referred to as spherulitic, even if the overall morphology is not a sphere [295].

Spherulite formation is not specific, and can be shown to occur in both of the primary mineral constituents of stones. For example, Figure 4.39 shows CaOx spherulites formed *in vitro*, and Figure 4.40 shows examples of CaP spherulites. Given that the overall morphology of a spherulite is usually a sphere, regardless of its composition, it is not easy to identify the crystal phase from visual examination, such as with distinguishable facets that occur in single crystals (e.g. the bipyramid shape of COD). However, sometimes the shapes of the polycrystals that make up the spherulite can help to identify its composition, but to be certain, verification with another technique, such as diffraction or spectroscopy, is required.

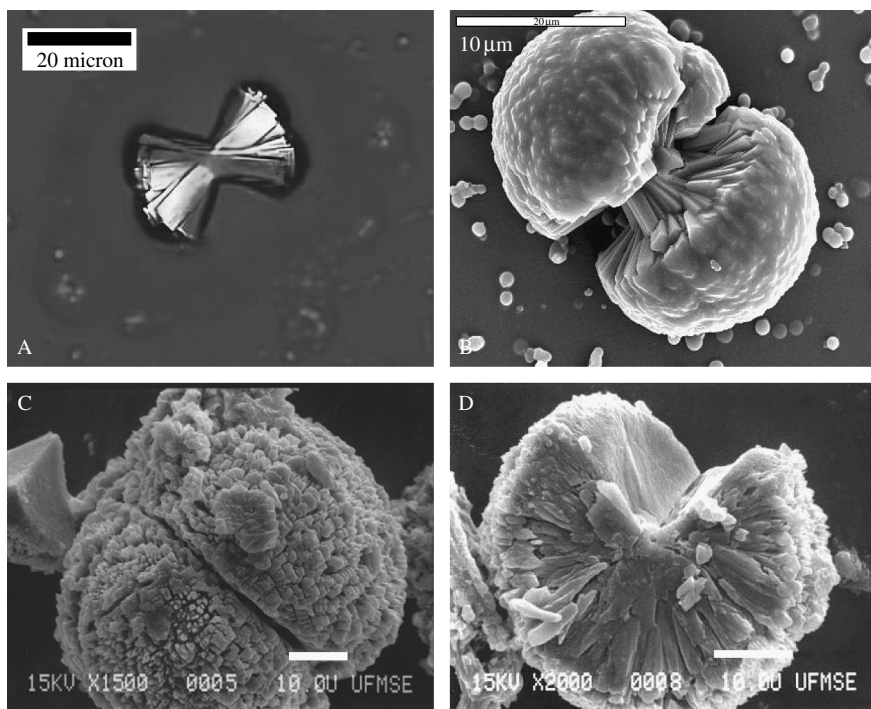


Figure 4.39 (Plate 7) Spherulitic morphologies of calcium oxalate. (A) Partial spherulite of 'sheaf of wheat' morphology. The shape of the individual polycrystals is flat platelets, which are most likely COM crystals. (B) Partial spherulite of 'dumbbell' morphology. The small round particles surrounding the sphere are 'droplets' of PILP phase. (C) The polycrystals have branched all the way around to form a double lobed spherulite. (D) Fracture surface of a full single lobed spherulite, showing the radial texture of the densely packed polycrystals.

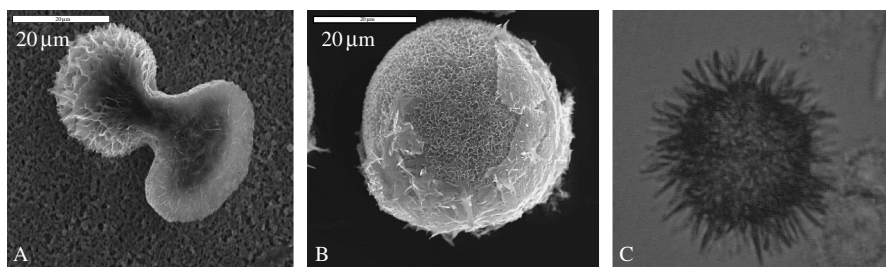


Figure 4.40 (Plate 8) Spherulitic morphologies of calcium phosphate. Note, platy clusters of HA can also grow from ACP, as shown in Figures 4.14 and 4.24A of the Bone section. (A) Partial spherulite of 'dumbbell' morphology. The shape of the individual polycrystals of the left bell appears to be irregular platelets, as is typical of the HA clusters mentioned above. (B) Full spherulite composed of densely packed polycrystals. The surface appears to be transforming into larger platelets. (C) 'Pin cushion' spherulite composed of needles (probably HA). These spherulites originated from smooth amorphous looking globules (such as at the bottom right), which apparently underwent surface dissolution and recrystallization into the more typical inorganic habit of needles.

As we stated earlier, the supersaturation is not excessively high in the urinary tract under normal conditions, but nonclassical nucleation of spherulites can also be brought about by a 'nucleation catalyst'. For example, this could simply be a microscopic impurity that blocks growth and stimulates secondary nucleation [292], or it might result from some type of specific nucleation promoter, which has a surface activity that is favorable for multiple nucleation events. Alternatively, it could also be the case that an inhibitor species, such as an acidic polymer, sequesters ions efficiently to raise the local supersaturation beyond the classical nucleation regime. Even inorganic impurities can poison crystal nucleation or growth, which is readily seen for the case of Mg ion, which leads to spherulitic CaCO_3 morphologies. Viscosity is also known to be a factor in stimulating spherulites in organic species [292,296], but it may also be relevant to minerals formed by a viscous amorphous precursor phase.

It is unfortunate that the stone literature rarely makes the distinction between a spherulitic type of aggregate vs aggregation of preformed crystals, because both appear to occur in stone formation, yet are formed by entirely different mechanisms. As can be seen, the spherical shape is quite easily distinguishable from a random aggregation of crystals (such as the CaOx crystals in Figures 4.34C and 4.41A), and the radial growth of the polycrystals can be easily identified by SEM of a fractured stone (Figure 4.41B), or by the Maltese cross pattern in polarized light microscopy of thin samples.

Amorphous Precursors to Spherulites

The possibility of amorphous precursor phases has not received the same attention as their crystalline counterparts, although ACP has been extracted from urine [290,297]. Amorphous calcium phosphate is not likely to be observed directly in stones because

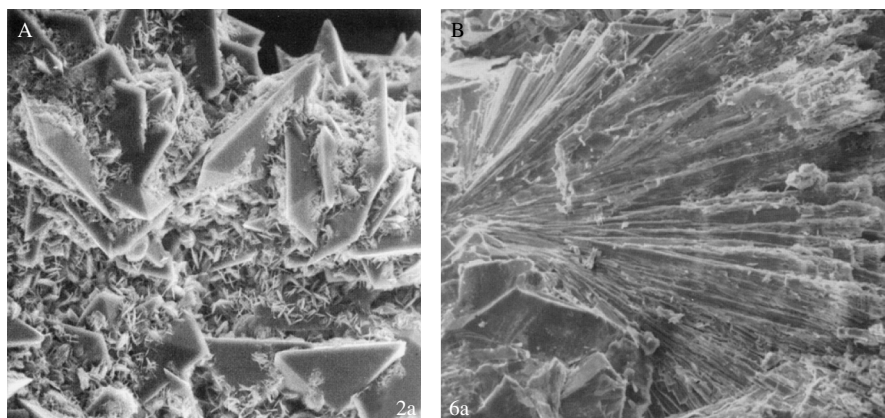


Figure 4.41 Scanning electron micrograph of urinary stones. (A) This stone appears to be a true aggregate, with rather flat bipyramidal crystals that are likely COD, randomly dispersed in a mesh of fine platy crystals (probably HA). (B) The radial growth pattern seen on the fracture surface of this calcium oxalate stone suggests that it is an ‘aggregate’ formed by spherulitic growth.

it will crystallize into the more stable form (such as HA) [298], especially if the calculi are retrieved at a later time when the problem is diagnosed and treated. Only a couple of papers have reported on the possible involvement of the less stable crystalline calcium phosphates, e.g. octacalcium phosphate (OCP) [294], as precursors in kidney stone formation.

We have observed spherulite formation *in vitro* to be a common outcome subsequent to the formation of amorphous phases, and in all of the calcific minerals we have examined (CaCO_3 , CaOx , CaP). Evidently the metastability of the amorphous phase results in dissolution and reprecipitation into a more stable phase, most often yielding a spherulite (Figures 4.8D and 4.40C). At this point, we can only speculate that the reason for transforming into a spherulite is due to the very highly localized concentration of ions released upon dissolution of the metastable precursor, which then stimulates rapid precipitation into the spherulitic cluster. Interestingly though, we have also seen evidence to suggest that not all spherulites form via dissolution–reprecipitation of the amorphous phase. Some spherulites appear to crystallize within the original amorphous globule (Figure 4.42). These spherulitic globules were apparently not a completely solid phase, because they seemed to ‘flow’ onto the substrate (Figure 4.42B). When examining the solution *in situ* in the very early stages of precipitation, we observed very faint spherical structures, with little to no birefringence. Amorphous (isotropic) materials form spherical objects to minimize surface energy, so it appears that in some cases, the spherical shape of spherulites is a direct result of the retention of this shape during the amorphous to crystalline transformation. This is particularly the case for the dense, smooth surfaced spherulites (Figures 4.39D and 4.40B), which have a distinctly different morphology than the loose, pin cushion

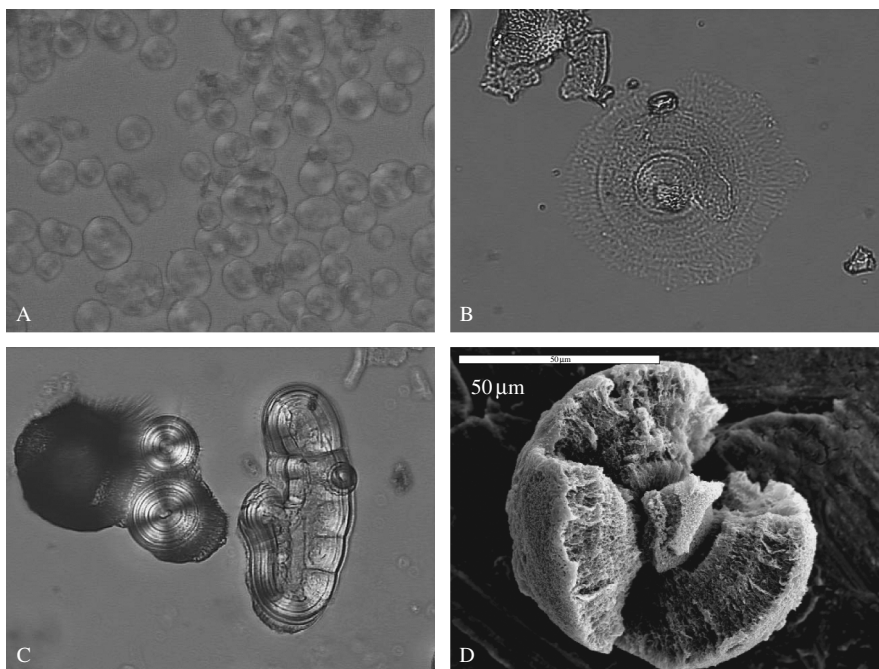


Figure 4.42 Transformation of amorphous CaP into spherulites. (A) When acidic polymer is added to the CaP synthesis, the spherulite precursors are somewhat different than the control; they appear to have a gelatinous consistency, smooth surfaces, and are initially very faint, becoming more brightly birefringent with time. (B) This early stage spherulite (evidenced by the Maltese cross texture) appears to have ‘flowed’ onto the glass substrate when it was dried. (C) Concentric laminations are conspicuous in these spherulitic agglomerates. The dark patches on the spherulites to the left are transforming to needles of HA presumably by a dissolution–recrystallization reaction. (D) SEM of this fractured spherulite shows the radial polycrystalline texture. Concentric laminations can be observed, as well as a dense ‘shell’. This spherulite apparently solidified via a pseudo-solid-state transformation into the more compact type of spherulite, which retains the rounded shape and smooth surface of the amorphous precursor (compared to pin cushions and platelets from recrystallization).

types of spherulites composed of individual needles (Figure 4.40C). Concentric laminations are frequently observed in spherulites formed from an amorphous precursor, as shown in Figures 4.42B–D, which we believe arise from exclusion of impurities during the crystallization of the precursor. This is observed in our *in vitro* model system using fluorescently labeled polyaspartate (Figure 4.43). The polymeric process directing agent is seen to be expelled to periodic layers in PILP deposited CaCO_3 films, and to the outer periphery of spherulites, which apparently leads to a differently textured shell (such as the one seen in Figure 4.42D).

Another interesting feature is that the amorphous globules start off barely visible, and they transform from a weakly birefringent to a fully birefringent and opaque

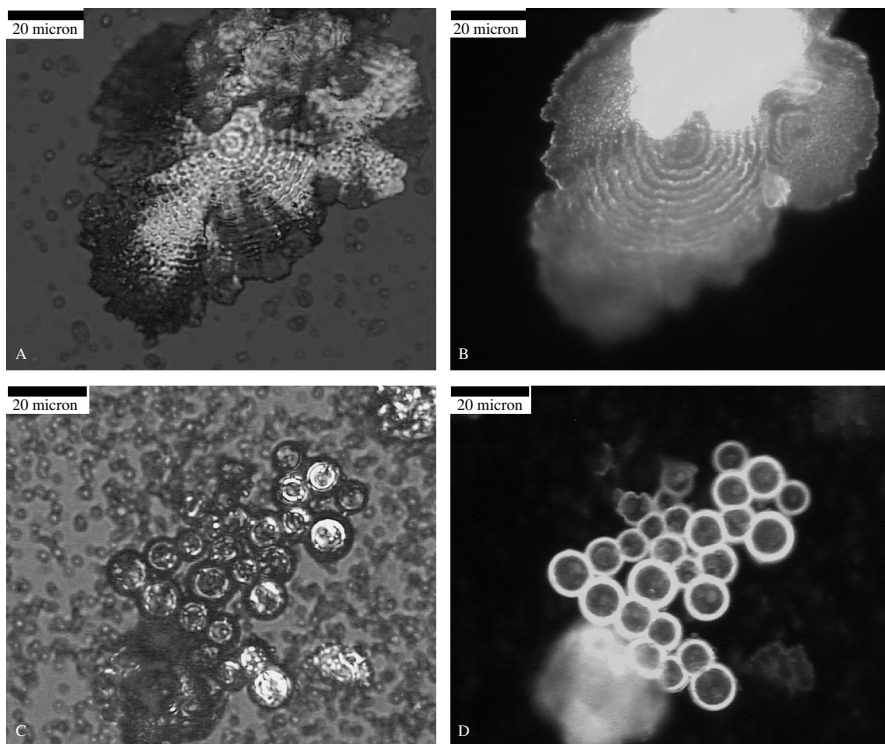


Figure 4.43 Exclusion of impurities during transformation of precursor phase. (A and B) This patch of spherulitic film was formed by the PILP process with CaCO_3 using fluorescently labeled process directing agent (FITC-polyaspartate). The concentric rings that are observed with cross polars in (A) are a result of exclusion of the polymeric impurity, as seen by the enhanced fluorescence of the ring. Note, the aggregate near the top is also very bright, supporting our argument that these aggregates are caused by a mixture of PILP phase with solution grown crystals. (C and D) These three-dimensional spherulites also try to exclude the polymer during crystallization, but apparently much of the polymer gets trapped towards the outer surface. The higher polymer concentration in the 'shell' region may then inhibit crystal nucleation/growth, leading to the more dense, fine grained texture in the shell region. Diffusional limitations are likely responsible for the regularity of the exclusion patterns.

sphere as they solidify with time (e.g., compare Figure 4.42A vs C, and fully solidified spherulites, which transmit very little light and appear brown). This was unexpected because, based on the traditional view of radial growth of crystallites from a central core, one would expect to see a pronounced birefringence in the center, which then progresses outwards as the material crystallizes. Instead, a uniform and gradual transformation of the birefringence across the whole sphere was observed. This seems to suggest the crystallization proceeds via a pseudo-solid-state transformation.

4.3.3 MIXED COMPOSITE STONES WITH CaP CORE

The occurrence of spherulitic calcium phosphate at the core of a number of kidney stones indicates the participation of the CaP mineral in the genesis of many kidney stones [289]. It has been proposed that calcium phosphate initially forms at the early segments of the nephron where supersaturation of calcium phosphate is high [299]. As the calcium phosphate precipitates (either amorphous globules or crystallites) travel through the collecting ducts, calcium oxalate crystals then become added to the stone because the supersaturation of calcium oxalate becomes high in these regions [300]. The CaP core could either induce heterogeneous nucleation of calcium oxalate onto the core, or could stimulate aggregation with CaOx crystals which are present in the surrounding medium [229,230]. Either mechanism could lead to a CaP core with CaOx crystals at the periphery, but the morphology of the CaOx crystals could provide additional clues to this stage of stone formation.

Mimicking Mixed Composite Stones

The complexity of the environment where stones are formed has hindered the ability to model such processes *in vitro*, and particularly the mixed composition stones. Urine, for example, is supersaturated with ions such as calcium, oxalate and phosphate, and various combinations of these can form the inorganic component of the kidney stone [299,300]. However, the supersaturation of these ions varies as urine flows through different parts of the nephron. Phosphate is highly supersaturated in the earlier segments of the nephron such that the formation of calcium phosphate is likely to occur in these regions [299]. Meanwhile, calcium oxalate forms in the distal tubules and the collecting ducts of the renal papilla where the supersaturation of oxalate is high [300]. Even bicarbonate is thought to be supersaturated in the loop of Henle, and the formation of calcium carbonate was proposed [299,301]. Thus, in order to fully mimic formation of kidney stones, different solutions with varying degrees of supersaturation would need to be employed [302,303], which along with the many impurities present in urine, present a huge challenge towards studying stone formation. We have approached this task by first examining one aspect of this process, how CaOx crystals form in the presence of a CaP core particle.

The calcium phosphate core in our model system is in the form of an amorphous spherical particle produced by the PILP process (Figure 4.44). These spheres have diameters as large as 0.6 μm , although smaller particles have been observed in these systems. Energy dispersive spectroscopy (EDS) on these particles reveal both Ca and P peaks (Figure 4.44B). XRD of these particles reveals no sharp diffraction peaks, as would be expected for an amorphous phase.

These amorphous CaP particles were placed in a solution that was gradually raised in supersaturation with oxalate ions through exposure of the solution to oxalic acid vapor. This results in a decrease in the pH of the solution. It also results in the dissolution of the particles, which is evident after 1 day, where the amount of the initial precipitates is decreased. During the pH drop, the particles seem to

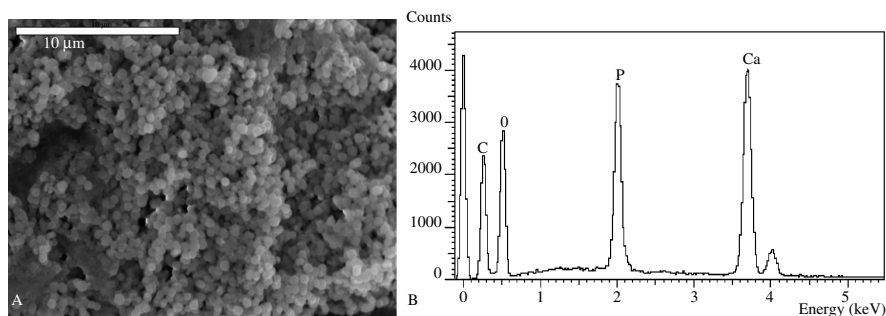


Figure 4.44 Scanning electron micrograph of amorphous CaP particles used as the core of our mixed composition mimetic stones. (A) The diameter of the spheres is approximately $0.6\mu\text{m}$. (B) Energy dispersive spectroscopy of the sample shows Ca and P peaks (the carbon peak is due to the carbon coating used for sample preparation), while XRD shows no diffraction peaks (data not shown), indicating the spherical particles are amorphous and not spherulites.

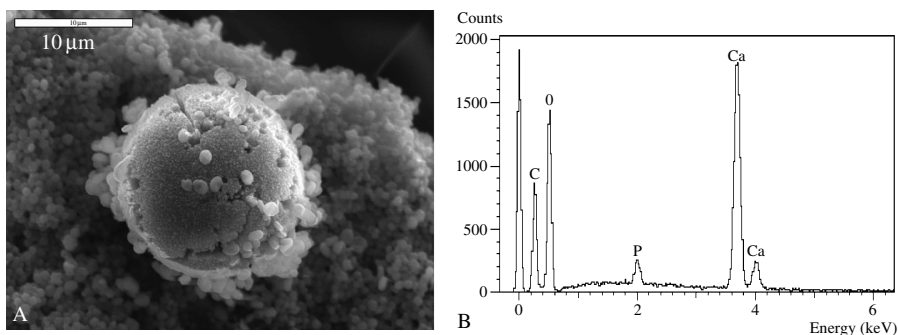


Figure 4.45 Influence of oxalate overgrowth on ACP particles. (A) Scanning electron micrograph of coalesced ACP particles during the early stages of oxalic acid vapor exposure. The particles in the background are the size of the initial ACP starting material. (B) The reduced phosphorous peak in the energy dispersive spectrum suggests that calcium oxalate is being incorporated during coalescence of the primary ACP particles, either as a mixture of ionic species in an amorphous phase, or as a CaOx coating.

coalesce into bigger structures, as seen by the formation of the $15\mu\text{m}$ diameter sphere shown in Figure 4.45A. Interestingly, the aggregation of amorphous CaP was only observed when a polymeric additive, such as poly(aspartic acid), was present. Without this polymer, the amorphous CaP completely dissolved without coalescence during the addition of oxalic acid vapor. The acidic polymer seems to serve as an aggregating agent that binds the amorphous CaP particles together, while stabilizing them against total dissolution.

A layer of calcium oxalate may have deposited on or within the aggregated amorphous CaP since EDS of this structure showed a decrease in the phosphorous

peak (Figure 4.45B). With further increase in the supersaturation of oxalate ions in the solution, a pronounced spherulitic overgrowth of calcium oxalate was observed on the core structure (Figure 4.46A). Not all of the spherulites, however, show this distinct nidus (Figure 4.46B). The calcium oxalate spherulites produced by this overgrowth technique were in the form of calcium oxalate dihydrate (COD), although calcium oxalate monohydrate (COM) spherulites also formed in different experiments. The polycrystals comprising the spherulitic overgrowth were quite large with facets that appear to be from tetragonal bipyramidal prisms, which are characteristic of COD. Figure 4.47 shows the X-ray diffraction pattern of the composite, with spacings consistent with the COD phase.

The spherulitic structures observed in this experiment are reminiscent of certain types of naturally occurring kidney stones, that is, those having a calcium phosphate core and calcium oxalate periphery [203,232,290]. Most of the cores in these kidney stones, however, are hydroxyapatite and not amorphous CaP. We do not know if that is true of our sample at the later stage because it is not easy to determine the crystal phase of the core once the overgrowth reaction is completed. HA was not detected by XRD, but it is not clear if there is sufficient crystalline material to resolve the peaks of this minor phase, or if it was still amorphous at this time stage. However, we do know from separate experiments involving the formation of ACP only (without overgrowth of CaOx), that the ACP transforms into hydroxyapatite under conditions similar to those employed here. Therefore, we predict that this transformation will likely occur (at some point) in the core of overgrowth composites as well.

The close semblance of certain kidney stones with our synthetic composite prompts us to propose this as a possible growth mechanism for the mixed kidney stones. In this model (see schematic in Figure 4.48), the formation of a kidney stone

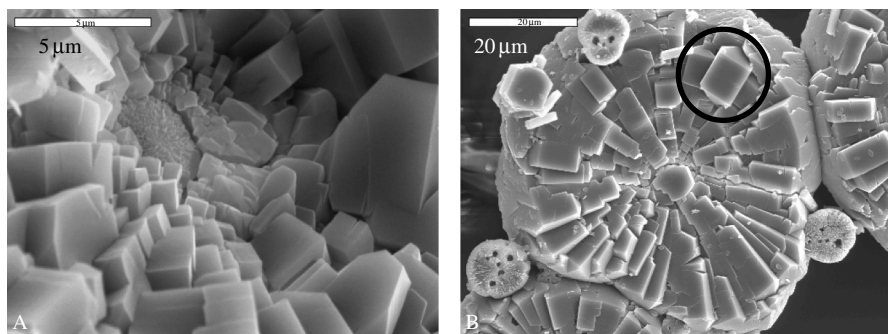


Figure 4.46 Calcium oxalate spherulites with differing core structures produced by overgrowth on ACP particles. (A) The core has a distinctly different texture than the surrounding blocky crystals, and is either still amorphous CaP, or very fine grained crystalline phase. (B) There appears to be a core in the center of this spherulite as well, but it has a more smooth texture being similar to the surrounding crystals. The circle marked in shows a tetragonal bipyramidal prism characteristic of calcium oxalate dihydrate, which was confirmed by XRD.

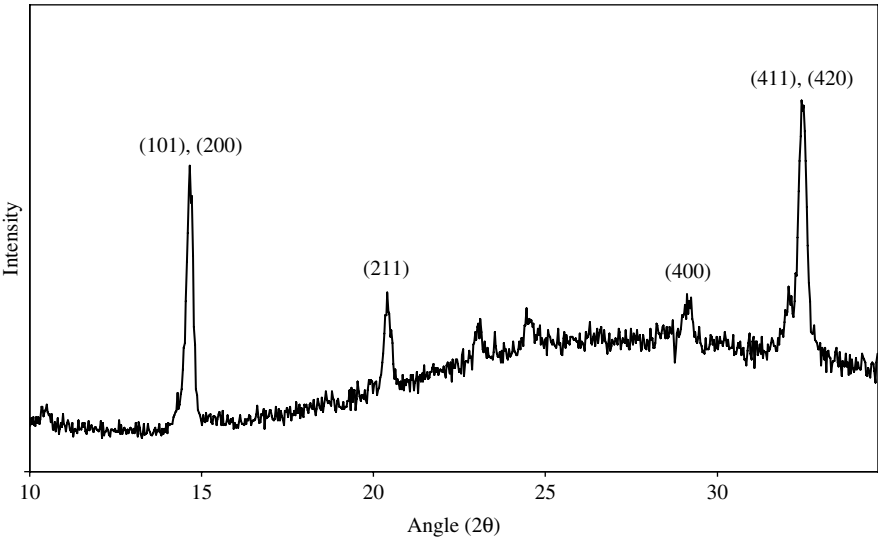


Figure 4.47 X-ray diffraction pattern of the calcium oxalate dihydrate spherulites.

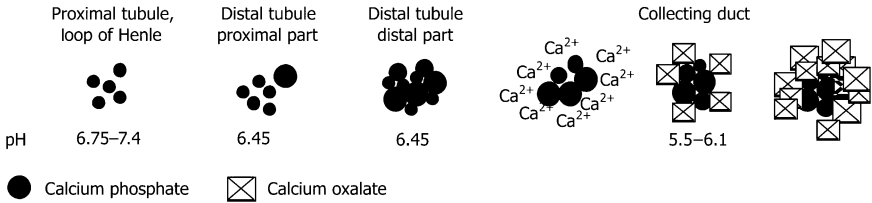


Figure 4.48 Proposed mechanism for the formation of CaP-CaOx mixed kidney stones. (Reproduced by permission of Springer-Verlag from Hojgaard I, Tiselius HG: Crystallization in the Nephron, *Urological Research* 27: 397–403 (1999).) In the early segments of the nephron, where urine is supersaturated with CaP, particles of CaP (either amorphous or spherulites) nucleate and grow. Once these CaP particles reach the collecting duct, where pH is relatively low, partial dissolution of the CaP particles occurs, being stabilized and agglomerated by the macromolecular constituents in the urine. Also at this region, the urine is supersaturated with CaOx, thus CaOx nucleates heterogeneously on the previously formed or reforming CaP particles, forming a mixed stone with a CaP core-CaOx periphery.

is initiated by the precipitation of ACP, which is suggested to occur in the loop of Henle [299]. As the urine containing the ACP particles traverses the tubules, it experiences an increase in the supersaturation of oxalate, and a drop in pH of the solution, such that heterogeneous nucleation of CaOx occurs. It is known that there is an increase in the supersaturation of oxalate ion and the acidity in the urinary environment during the transit from the proximal tubules to the distal tubules of the collecting ducts [299,300,302,303]. The presence of macromolecules and

organic debris in urine may serve as stabilizers for amorphous CaP in the acidic media, as we observed in our *in vitro* studies involving Pasp. Instead of completely dissolving, the ACP might coalesce into bigger particles that serve as the core for spherulitic growth of CaOx. At some point, the amorphous CaP core transforms into the more thermodynamically favored HA, leaving behind evidence of only an HA core.

It should be noted that epitaxial overgrowth is not considered a mechanism for the growth of CaOx in our model, because there is no lattice matching between mineral species when the initial CaP phase is amorphous. We cannot definitively say that the ACP core did not transform into a crystalline phase right before the crystallization of CaOx, but there is no evidence to indicate that this had occurred. We can conclude, however, that there is some type of ‘templating’ effect of the CaP core on the growth of CaOx, because different final configurations of the CaOx crystals were induced by its presence.

As is true with most *in vitro* models, this is most likely an oversimplification of the actual growth of kidney stones because many factors have not been included in the system, and it seems that many types of stones are formed. Nevertheless, we present this model to suggest an alternative hypothesis regarding the interaction of calcium phosphates and oxalates through an amorphous precursor intermediate.

4.3.4 MORPHOLOGICAL CLUES OF CRYSTALLIZATION MECHANISM

Morphological features of the crystals within a stone could be providing clues as to the mechanism, and therefore the reason, for stone formation. Some features that could be identified include the arrangement of the crystals. Are they adsorbed in a random arrangement, such as might occur from adsorption and aggregation of preformed crystals (Figure 4.41A), or is there radial orientation of the crystals from some central core (Figure 4.41B)? It would be of value to know what the composition and structure is of such a core, which seems to be significant in instigating stone formation. Many stones contain concentric laminations of mineral phase (Figures 4.31B, 4.31C, 4.33 and 4.36A), often with organic matrix inbetween. This might be expected to arise from episodic changes in the urinary environment, such as dietary or dehydration, both of which could lead to a change in the degree of supersaturation. Once again, do the concentric mineral layers appear to be dense films (such as in Figure 4.31C), suggestive of a coating of an amorphous phase? Are the crystallites radially organized in concentric layers, suggestive of a spherulitic growth pattern with excluded impurities? If the spherulite has tightly packed polycrystals that contain little to no intercrystalline space, our observations thus far would lead us to speculate that they are formed from transformation of an amorphous precursor. Even the loose packed faceted spherulites often come from an amorphous precursor via the dissolution–recrystallization route, so a further understanding of crystallization via amorphous precursor phases could be of value.

Another aspect of the PILP process that could be relevant to stone formation includes the aggregation tendency of crystals formed in the presence of PILP phase. This was noticed early on during our studies with the CaCO_3 PILP system, that not only were mineral films formed, but aggregates of crystals were usually present as well (Figure 4.5D). Quite often the morphology of the single crystals comprising the aggregate would be faceted, but heavily distorted, suggesting that these crystals were hybrid crystals, predominantly formed by the traditional solution crystallization process, but modified extensively by the presence of the fluidic precursor (Figure 4.5A). While the exact cause of this aggregation tendency is not known, it seems reasonable to conclude that any process that leads to pronounced aggregation tendencies could be a factor in stone formation, and especially when it is triggered by acidic polypeptides. Morphological clues of this hybrid condition might include aggregates containing rounded crystals, or faceted crystals with 'molten' distorted habits.

The PILP process leads to both types of aggregates, the spherulitic clusters discussed earlier, as well as the aggregation of already formed solution crystals. As an example, in preliminary studies on the formation of a calcium oxalate PILP phase (Figure 4.49), both types of aggregates can be seen in the polarized optical micrographs. The Maltese cross pattern of the central sphere of Figure 4.49C indicates that a spherulite of CaOx has formed, while the surrounding particles are solidified PILP droplets which appear to be aggregating around this central spherulite. Similar aggregation tendencies of PILP droplets have been observed in the CaCO_3 and CaP PILP systems as well.

The stone structures containing concentric laminations have intrigued us because one might anticipate that the more typical faceted CaOx crystals would be deposited on the surface of the growing stone during episodic growth stages. Certainly in some stones, morphologies typical of COM (coffin shaped) and COD (bipyramids) can be readily discerned. But these concentric laminations bring to mind the sequential deposition of mineral coatings. In light of our *in vitro* studies on PILP coatings, this is a possibility. The mineral films that are formed by the PILP process could certainly be envisioned as leading to such concentric coatings, and as a rough demonstration of this concept, we generated a CaOx PILP phase to deposit a CaOx 'coating' onto a preformed crystalline CaP spherulite (Figure 4.50A). One can imagine that if such PILP forming conditions were to episodically occur in the urinary tract, that mineral coatings could build up a concentrically laminated stone. We expect that much of the organic matrix (such as the polymer that induced the mineral precursor) would be excluded toward the periphery of each layer as it solidifies, as mentioned above (Figure 4.43). Even without a fluidic PILP phase, there appears to be pronounced aggregation of small CaOx particles onto a CaP spherulite (Figure 4.49B). Once again, this type of deposition process should lead to a different microstructure than deposition of a mineral coating.

Other experiments in our group have found that the PILP phase can have quite a pronounced tendency to preferentially deposit on mineral surfaces, or charged and hydrophilic organic substrates, as opposed to hydrophobic or less polar substrates

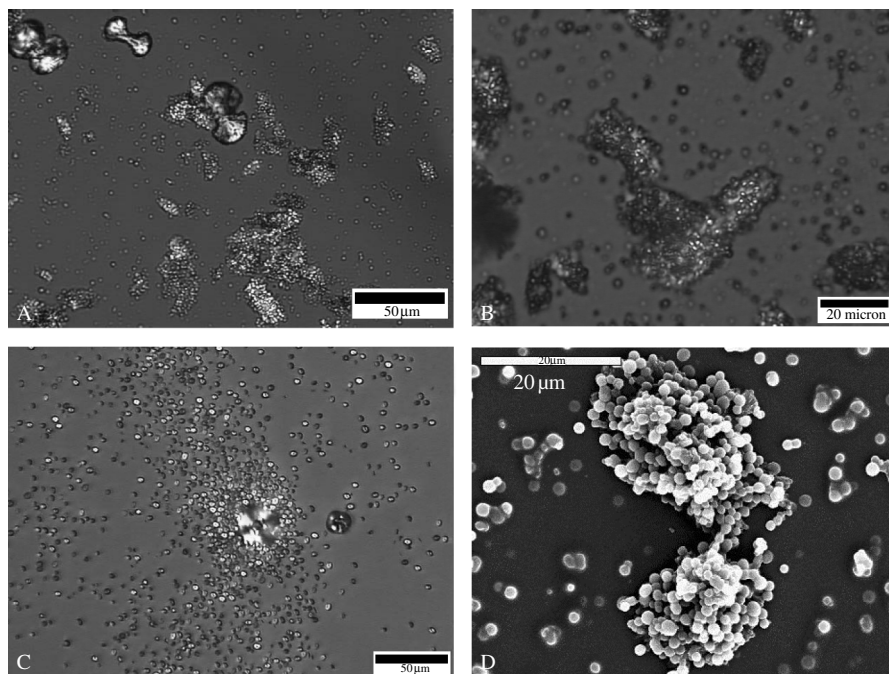


Figure 4.49 Formation of calcium oxalate by the PILP process. Figures A–C are optical micrographs using cross polarized light with a 1st order red γ plate. (A) An amorphous precursor film covers the glass coverslip, but being isotropic, it is not readily detected since it matches the background. However, it can be partially discerned by the bumpiness from more solidified droplets that did not fully coalesce with the film. The presence of the film becomes more obvious as it transforms, at which point birefringent orange and blue patches spread across the film as it crystallizes. The large structures in the upper left of the picture are dumbbell shaped spherulitic aggregates. (B) This higher magnification view shows a close-up of the birefringent patches of film. The bumps indicate that the PILP droplets were approximately $2\text{ }\mu\text{m}$ in diameter, similar to those observed in the CaCO_3 system. (C) These isolated PILP droplets are now crystalline, as evident by the birefringence. Note the rather striking tendency of the droplets to cluster around the spherulite in the center of the photo (with the Maltese cross pattern). (D) SEM of the PILP droplets showing a tendency to aggregate into small clumps, which did not coalesce as well as the surrounding film). It can be seen that the individual crystal ‘drops’ are quite round, and not faceted.

(e.g. patterning ability shown in Figure 4.7C). It is these sorts of interfacial interactions, which we would consider to be ‘nonspecific’, that may explain why certain organic species are preferentially assimilated into stones. But presently, this discussion is mostly speculative, and further work is needed to clarify how the PILP phase interacts with cells and organic constituents of the urinary environment. At this point, the goal here is to illustrate some of the physical features that may be relevant to kidney stones, which if examined from a materials chemistry perspective, could be providing clues as to their mechanism of formation.

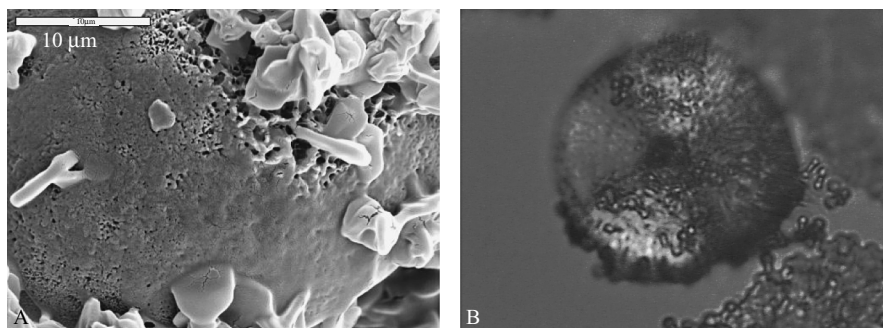


Figure 4.50 Deposition of CaOx on preformed CaP spherulites in the presence of polyaspartate. (A) This SEM micrograph shows a film like coating that deposited on the surface of the spherulite, which we believe was from CaOx PILP phase induced by the polymer. There are also aggregates of small ill defined crystals, which appear to also have been influenced by the polymer. The rough texture of the underlying CaP polycrystals of the spherulite can be seen, which clearly did not require an epitaxial match to stimulate CaOx overgrowths. (B) This polarized light micrograph shows aggregation of many small particles as well as adsorption of the CaOx particles to the surface of the CaP spherulite. The small particles are birefringent and appear to be crystalline dumbbells at this stage, which cannot coalesce into a mineral coating, as in (A).

4.3.5 CONCLUDING REMARKS ON STONE FORMATION

Many of the proteins extracted from the organic matrix of calcific crystals are known to be modifiers of crystallization, whether it occurs during crystal nucleation, growth or aggregation. In the case of stone formation, all of these stages may be important in the formation or retention of the stone. In the case of biologically-induced mineralizations, such as stone formation, one would assume that most of the proteins are not genetically designed to have a 'specific' interaction with the crystal, but it is possible that some proteins are secreted by the renal epithelial cells for the specific function of inhibiting crystal nucleation, growth or aggregation [304,305]. In other words, these functions most likely do not require a high degree of molecular recognition between the protein and crystal, since there are many crystal types and crystal faces present during stone formation, but instead require blocking capabilities that can likely be achieved more rapidly and efficiently with adsorption of 'nonspecific' additives (unless molecular recognition provides a more potent antidote to the problem). Our present thoughts on this (which are still in the speculative stage), are that these nonspecific inhibitory proteins may be secreted by the renal epithelial cells, most often resulting in successful inhibition of the stone; but under certain conditions, the desired 'inhibitory' action of the protein is overcome by the reaction conditions, leading to amorphous precursors. As we have tried to demonstrate in this overview, the presence of amorphous phases, which might be very useful in biologically controlled mineralizations, could have detrimental effects in pathological mineralizations, leading to some of the features observed in

stones, such as spherulites, core overgrowth structures, concentric laminations and generalized aggregation tendencies.

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5 Pathological Biomineralization of Iron

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5.1 INTRODUCTION

5.1.1 BIOGENIC IRON OXIDES

The presence of iron oxides in various organisms ranging from bacteria through to humans illustrates both the importance and the complexity of the biochemical reactions involved in iron biomineralization processes [1–3]. Biological mineralization refers to the processes by which various solid phases are deposited in biological molecules, cells, and tissues [4]. One important class of iron biominerals is that of the iron(III) hydroxides or oxyhydroxides, which occur variously as amorphous, colloidal precipitates, as semicrystalline minerals such as ferrihydrite, or as crystalline minerals such as magnetite and goethite [3]. The term ‘iron oxides’ is used generally to embrace the iron oxides, hydroxides and oxyhydroxides [5]. The formation of iron biominerals allows organisms to accumulate iron for various functions, particularly those consistent with their metabolic needs. Iron oxide biominerals reported as present in humans include amorphous iron(III) oxyhydroxides, ferrihydrite ($5\text{Fe}_2\text{O}_3 \cdot 9\text{H}_2\text{O}$), poorly crystalline goethite ($\alpha\text{-FeOOH}$) and magnetite (Fe_3O_4) [3].

5.1.2 IRON METABOLISM

Iron is an essential metal ion for almost all organisms [6,7]. For example, it participates in a variety of electron transport pathways, oxygen transport and storage and, among other functions, is also required for the enzyme ribonucleotide reductase, which in turn is required for DNA synthesis and cell division [8]. While iron is required for many functional molecules and so is in constant use, at the same time some iron needs to be held in reserve for times when it is deficient. As such, the

storage of iron is a necessary feature of human iron metabolism. However, unbound iron is toxic and needs to be kept in a safe and nonreactive form such as protein bound iron. The presence of unbound iron is involved in the generation of toxic oxygen species leading to free radical damage in the tissue [9].

Under normal conditions, effective regulatory mechanisms ensure the presence of a sufficient amount of iron in the body. The amount of iron is regulated by control of iron absorption, while iron excretion is limited [10–12]. If iron is lost from the body, or if dietary iron intake is lowered, storage iron can be mobilized. If amounts of iron larger than are required to meet the small daily losses enter the body due to metabolic or physical disturbance, the excess must be stored, since the body has no specific mechanisms of excreting excess iron [11,13].

Iron absorbed from the diet is distributed throughout the body *via* the soluble plasma carrier protein, transferrin [14,15]. Iron enters most cells, particularly those with high iron requirements such as the erythropoietic cells in bone marrow, using the transferrin receptor which is a specific membrane protein with high affinity for iron(III) bound transferrin [16–20]. Once iron enters the cell, it moves into a metabolic active pool where it is partitioned between utilization for synthesis of essential cellular constituents and deposition in the iron storage compartments, ferritin and, under some circumstances, hemosiderin. The major sites of iron storage are the liver, spleen, and bone marrow. Any disturbance of the uptake of iron invariably leads to a disturbance of the overall iron balance of the body.

5.1.3 IRON OVERLOAD

Iron overload is commonly encountered in the several pathologies contained under the general terms thalassemia and primary (idiopathic) hemochromatosis [21]. The abnormally high levels of stored iron in thalassemia are a secondary effect arising from ineffective erythropoiesis (red blood cell production) [22]. Iron overload in thalassemia can also be accelerated if patients are treated with regular blood transfusions in the absence of effective iron chelation therapy (see later). In contrast, in the case of idiopathic or primary hemochromatosis, iron overload is a primary symptom of the disease in that there is a genetic defect (an autosomal recessive disorder) directly linked to iron uptake. Currently there are five different genes (*HFE*, hemojuvelin, hepcidin, transferrin receptor 2 and ferroportin) that are known to be affected by mutations that result in primary hemochromatosis [23]. Each of these mutations results in a disruption of the control of iron uptake by the human body. However, the rate of iron loading varies both between the different genotypes and also within a particular genotype.

Thalassemia comprises a group of diseases in which synthesis of hemoglobin is impaired, leading to a chronic anemia [24,25]. The anemia is due to an abnormality in hemoglobin production resulting from a variety of molecular defects that reduce synthesis of the α -like (α -thalassemia) or β -like (β -thalassemia) globin chains of hemoglobin [26,27]. The shortening of thalassemic red blood cell survival has been

found to be directly related to the degree of globin imbalance [28] leading to the rapid removal of defective red blood cells. The rapid removal of red blood cells leads to extensive destruction of both immature and mature red cells which results in chronic haemolytic anemia. In order to increase erythropoiesis, the erythroid marrow of patients with thalassemia major proliferates up to 10 to 20 times the normal cellularity [29–31]. The increased marrow iron requirements for this greatly increased red cell production results in greatly increased iron absorption. As such, a consequence of this chronic anemia is a progressive increase in iron loading and accumulation [21,32].

Thalassemic and primary hemochromatotic diseases are global in their distribution. The prevalence of the homozygous state for *HFE* related hemochromatosis is approximately 5.4 per 1000 asymptomatic subjects in various populations [33]. Various forms of thalassemia are common in a vast area from the Mediterranean region through to Asia (Figure 5.1). In Southeast Asia, the reported gene frequencies are as high as 30 % for α -thalassemia, 3–9 % for β -thalassemia and up to 50–60 % for hemoglobin (Hb) E at the junction of Thailand, Laos and Cambodia [34,35]. Thalassemias are very heterogeneous in molecular defects with different molecular mechanisms, most of which are base substitutions or small deletions of one or two nucleotides [36]. For example, Hb E results from a mutation at position 26 of the β -globin chain (glu $\rightarrow$ lys) [37]. Different combinations of these genes result in a spectrum of over 60 thalassemia syndromes ranging in severity from asymptomatic to fatal [34,35].

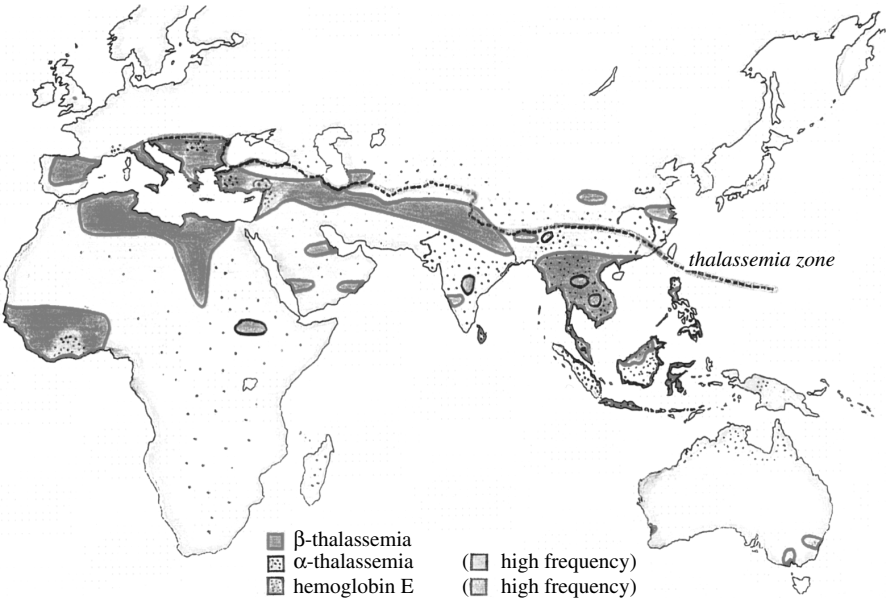


Figure 5.1 (Plate 9) Global distribution of thalassemic genes.

Pathology and Treatment of Iron Overload

Increased iron absorption or parenteral iron administration lead to excess iron accumulation in various organs, particularly the liver, spleen, pancreas and heart. [38–41]. Cellular damage and functional insufficiency of organs involved are related to the progression of iron deposition. Excessive intracellular iron may damage the cell by different mechanisms such as iron induced peroxidative injury to organelle membranes [42] or iron stimulated depletion of vitamin C and E stores [43]. Despite these studies, the mechanism of the iron toxicity in iron overload is still not known in detail. A significant fraction of nontransferrin bound iron in plasma is found in both thalassemic and hemochromatotic conditions [44–46]. This iron fraction has been suggested as a major source of free radicals that may well be the major agent responsible for tissue damage [46].

The removal of excess iron is the major focus in the treatment of iron overload. In the case of hereditary hemochromatosis, phlebotomy (the removal of blood) is the treatment of choice and is very successful in ameliorating problems associated with this genetic defect [38]. However, in thalassemia the situation is complex since the excess iron is not only derived from intestinal absorption but also from catabolism of red cells and degradation of haemoglobin [32]. Thus, phlebotomy cannot be employed owing to the inherent anemia associated with thalassemia. In order to eliminate the complications of anemia and compensatory bone marrow expansion, regular red blood cell transfusions have been introduced as part of the treatment. Iron absorption can be limited if red cell production is inhibited by a suitable transfusion protocol capable of keeping hemoglobin at normal levels [47,48]. However, transfusions result in the inexorable accumulation of tissue iron as the transfused cells are eventually removed from the circulation [49]. Thus, the blood transfusion regimen is frequently associated with regular chelation therapy. In the absence of chelation therapy, an option to reduce the degree of iron loading is to diminish or minimize the transfusion intake. This option has been adopted in Southeast Asian countries where a high prevalence of the disease occurs and where regular blood transfusions are not generally available [34].

In thalassemic patients who receive regular blood transfusions, chelation therapy has been used to complex the excess iron and promote its excretion. The subcutaneous infusion of desferrioxamine (DFO) is the primary method of eliminating large quantities of iron with the results giving a proven clinical benefit [50,51]. Such treatment has been shown to produce negative iron balance and to prolong life expectancy with low long term toxicity in iron overloaded patients [47,52].

Desferrioxamine (DFO) is derived from *Streptomyces pilosus* and is a hexadentate hydrophilic iron chelator consisting of three hydroxamic acids and a terminal amino group with an overall molecular weight of 657 Daltons. DFO interacts with iron by forming a high affinity complex with a formation constant of 10^{31} [53]. The selectivity for iron(III) is remarkably high with the molecule forming a hexacoordinate octahedral complex with a 1:1 stoichiometry [54]. In the normal state, most of the iron in the body is unavailable for DFO chelation. For example, hemoglobin iron, representing more than two thirds of all iron in the normal body,

is unavailable for DFO chelation either *in vivo* or *in vitro* [53]. Similarly, transferrin bound iron is a very poor source of iron for *in vivo* chelation by DFO even though the stability of DFO with iron(III) exceeds that of transferrin. As such, the most likely source of chelatable iron is either that stored in the tissues in the form of ferritin or hemosiderin or that in a labile iron compartment in the cell which is in dynamic equilibrium with iron stored in ferritin [55].

At the time of going to press, there are several oral iron chelators in various stages of clinical testing. Deferiprone, which has health authority regulatory clearance in several jurisdictions as a second line therapy for those patients who cannot be treated effectively with DFO, is the most extensively clinically tested of these oral iron chelators [56]. Three deferiprone molecules are required to bind one iron atom (bidentate chelation). Each molecule is neutrally charged and has a molecular weight approximately one third that of desferrioxamine and is more lipid soluble. These properties allow rapid gastrointestinal absorption and access to intracellular iron pools [57] and may possibly lead to enhanced chelation of iron from the heart [58].

5.1.4 IRON OXIDE DEPOSITS IN TISSUES

Mammals usually store iron in tissues such as the liver and spleen. In both thalassemia and primary hemochromatosis, excess iron is deposited in the tissues in the form of ultrafine particles of iron(III) oxyhydroxide [59]. At low levels of loading, the iron(III) oxyhydroxide particles are mostly found within the water soluble iron storage protein, ferritin. At higher levels of iron loading, iron(III) oxyhydroxide particles occur mainly as insoluble aggregates associated with organic residues. This form of deposit is known as hemosiderin [60–64].

Ferritin

The iron storage protein ferritin provides an example of biologically controlled mineralization. The formation of the iron core in ferritin involves highly controlled mineralization and the resulting particles often have a very narrow size distribution [65]. Ferritin is an approximately spherical molecule with a central cavity within which the iron(III) oxyhydroxide particle is held (see reviews by others [59,66–70]).

Ferritin consists of 24 polypeptide subunits that are assembled into an approximately spherical cage with an outer diameter of about 12 nm and an internal diameter of about 8 nm (see Figure 5.2).

The protein shell, which is approximately 2 nm thick, renders the particle water soluble. Channels connect the internal cavity with the external space and facilitate the migration of ions both into and out from the internal cavity [71]. Iron can enter the cavity and precipitate in the form of an iron(III) oxyhydroxide particle. Within the central cavity, variable amounts of iron can be accommodated although the size of the resulting particle is limited by the dimensions of the ferritin cavity. While apoferritin contains no iron, a fully loaded molecule of ferritin can, in principle, contain up to 4500 atoms of iron(III) [68].

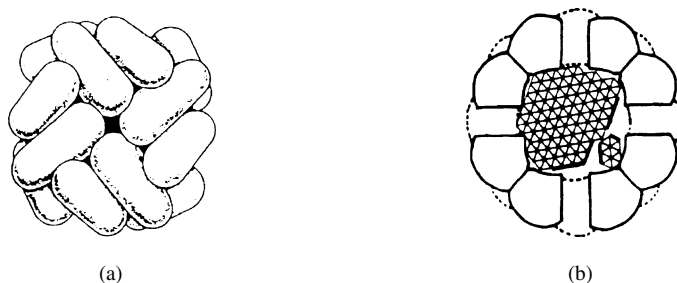


Figure 5.2 Schematic diagrams of ferritin: (a) The 24 self-assembled subunits making up the protein cage (adapted from [176]) and (b) A cross section through the protein cage showing the inorganic iron core (taken from [177]).

The iron sequestered within the central cavity of ferritin is present as one or more crystallites of the biomineral, ferrihydrite, with an approximate composition of $5\text{Fe}_2\text{O}_3 \cdot 9\text{H}_2\text{O}$ [59,69,72,73]. Varying concentrations of inorganic phosphate are also found within the biomineral core of ferritin [74]. The biomineralized iron forms a core which can be imaged using transmission electron microscopy (TEM) (see Figure 5.3).

The Mechanism of the Iron Oxyhydroxide Core Formation

Formation of the ferritin iron oxyhydroxide core is a multiple step process which has been reviewed previously [66,68]. The process firstly involves the transient binding of iron(II) to protein sites of the catalytic centre. Iron(II) is then oxidized to iron(III) by molecular oxygen or other oxidants. In the early stages of oxidation,

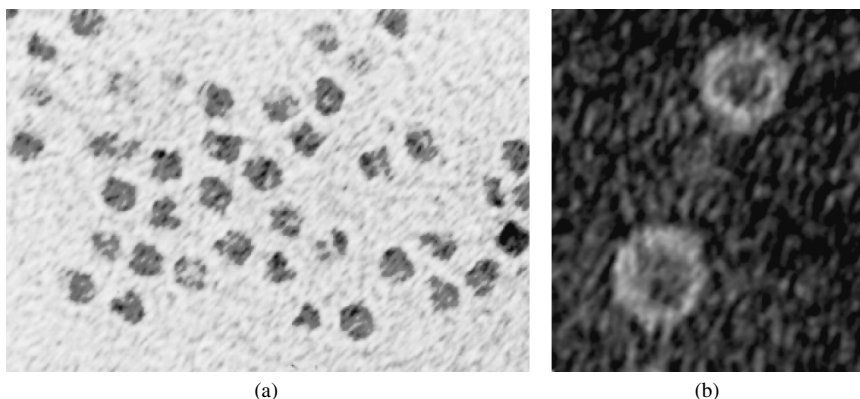


Figure 5.3 Transmission electron micrographs of human liver ferritin: (a) images of electron dense ferritin cores and (b) negative staining with phosphotungstic acid allows imaging of the low density protein cages around the electron dense cores.

an intermediate μ -oxo-bridged iron(III) dimer is formed [75,76]. Iron(III) dimers are then dissociated and replaced by monomeric iron(III) which migrates to the cavity [75–77]. Later, iron(III) clusters form in the cavity which yield stable nuclei of ferrihydrite. These nuclei provide a second surface onto which iron(II) can be deposited directly and then oxidized in a facile autocatalytic process [78]. As more iron(II) enters the cluster, the iron(III) atoms become magnetically coupled and the core particle size increases. These processes can occur simultaneously at the different sites in the same molecule or in different molecules [68]. More recently, evidence has been obtained for multiple pathways of mineral formation in mammalian apoferritin [79].

The Ferritin Iron Oxyhydroxide Core Structure

The structure of the iron core of ferritin has been principally studied by transmission electron microscopy (TEM), X-ray diffraction (XRD), electron diffraction (ED), Mössbauer spectroscopy, and to a lesser extent by extended X-ray absorption fine structure (EXAFS) spectroscopy and magnetometry [59,80–89]. Native cores in human ferritins show varying degrees of crystallinity ranging from noncrystalline to well crystalline [59]. The ferritin cores have a semirandom structure and a variable amount of phosphate present [59,74,90–93]. In well ordered cores, the mineral exhibits X-ray and electron diffraction patterns similar to those of ferrihydrite. The recent development of electron nanodiffraction techniques has enabled single crystal diffraction patterns to be obtained from single ferritin biomineral cores [94]. Studies on commercially prepared horse spleen ferritin indicated a variety of crystal structures with different ferritin molecules encapsulating different minerals. It was found that the majority of the cores in the commercial horse spleen ferritin sample had a hexagonal structure somewhat similar to the major phase in the mineral ferrihydrite. However, several minor phases were present including some that were similar in structure to the iron oxides magnetite and hematite and also some composed of highly disordered material [94]. In general, each core consisted of one single crystal of one phase. It has yet to be shown whether a similar variation of crystal structures exists in ferritins isolated from single organisms and whether the minor phases present in the commercial sample were due to the commercial methods of ferritin purification and processing. Owing to its varying degrees of crystallinity, ferrihydrite is usually classified empirically according to the number of major diffraction peaks or lines and is often described as either two line or six line ferrihydrite. Well ordered cores have been reported to show up to 14 lines, 6 of which are prominent when viewed by X-ray diffraction and electron diffraction [86,95].

Ferrihydrite

Ferrihydrite is generally obtained as particles of nanoscale size that are characterised by high dispersion, poor crystallinity and low stability [96–98]. Owing to the lack of long range order for this material and its small particle size, several structural models have been suggested (see Figure 5.4).

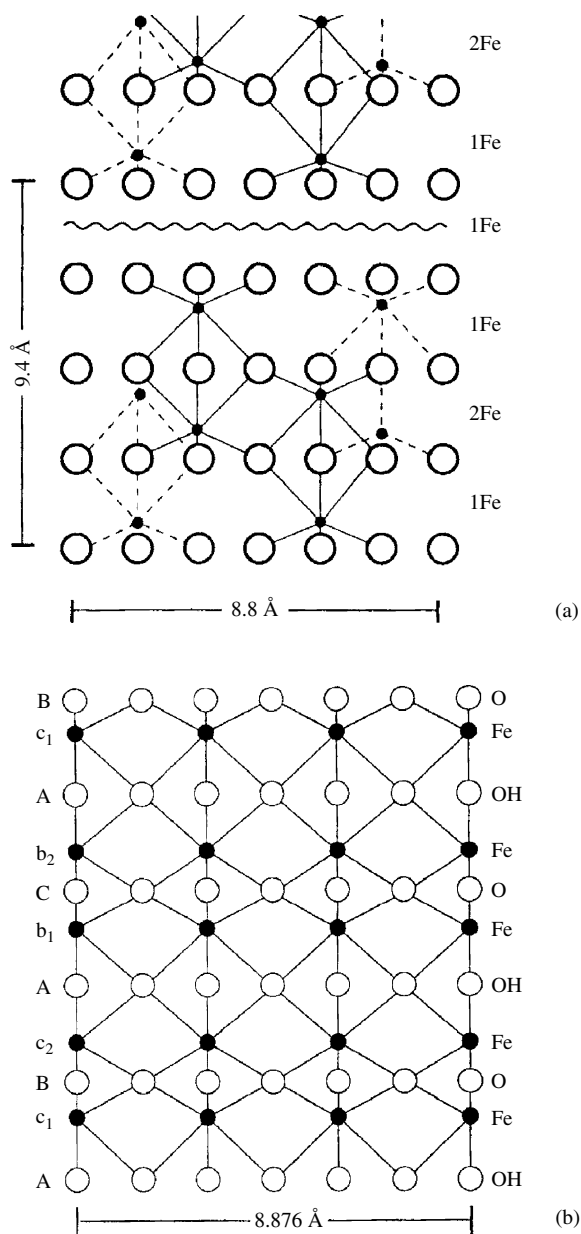


Figure 5.4 A comparison of the various models of ferrihydrite (○ : oxygen atoms; ● iron atoms): (a) ferrihydrite structure derived from that of haematite ($a = 5.03 \text{ \AA}$, $c = 13.74 \text{ \AA}$) having (1) a unit cell repeat of four, rather than six, oxygen layers along the *c* axis and (2) a variable occupancy of the metal within one of the four layers in each unit cell ([73]) and (b) regular displacement of iron atoms along the *c* axis toward B and C planes in which oxygen atoms prevail over hydroxyls. Only Fe octahedra in the (110) plane are shown. Occupancy of octahedra is 50 % ([99]).

The most widely accepted model is derived from the haematite (α -Fe₂O₃) structure [73]. In this model, ferrihydrite consists of hexagonally close packed oxygen planes with iron ions in octahedral interstices. The periodicity of the octahedral sheets along the z dimension is 4 for ferrihydrite ($c = 0.94$ nm) but 6 for haematite ($c = 1.3752$ nm) (Figure 5.4a). The low crystallinity of ferrihydrite is linked to the presence of vacant iron sites in the structure and to the replacement of some oxygen anions by H₂O and/or OH⁻.

More recent data with a modified structure model for ferrihydrite (Figure 5.4b) have been reported and allow its model to be reconsidered [99–101]. In this analysis, which is based on X-ray absorption spectroscopy and calculated X-ray diffraction curves, the results obtained from synthetic ferrihydrite indicate that it consists of three components. These are:

- (1) Structural anionic ABA and ACA fragments in which Fe cations occupy only octahedral sites; the cell is hexagonal, space group $P\bar{3}1c$ $a = 2.96$ Å, $c = 9.40$ Å. These fragments alternate in a regular pattern to form a three dimensional structure.
- (2) Fragments that alternate completely at random, but within which the Fe atoms have an ordered distribution in a hexagonal supercell with $a = 5.126$ Å.
- (3) Ultradispersed haematite in which coherent scattering domains are of the order of 10–20 Å.

The haematite accounts for the asymmetry of the X-ray diffraction peak at about 2.7 Å.

The main structural difference between six line and two line forms of ferrihydrite is the size of their coherent scattering domains, which are extremely small for the two line structure. These general structural features of ferrihydrite have recently been confirmed with neutron diffraction measurements [102].

In general, ferrihydrite is thermodynamically unstable and, with time, can transform into more kinetically stable forms. Which compound forms under a given set of conditions appears to be governed by kinetic factors such as pH, temperature, oxygen, ionic strength, the presence of ligands or organic molecules and other elements in the system [103,104]. It is important to note that the protein shell of ferritin does appear to be specific to the formation of ferrihydrite without phase transformation reactions. The mineral forms, α - or γ -FeOOH (goethite or lepidocrocite, respectively), can be produced under identical conditions for ferrihydrite ferritin core formation in the absence of the ferritin shell [89].

Hemosiderin

Hemosiderin was first identified under the optical microscope as aggregates of iron containing materials which gave a positive Prussian blue (Perls' reaction) stain [105]. The same aggregates can be imaged by electron microscopy as irregular, massive clusters of electron dense particles [41,106–111]. These aggregates are normally found within siderosomes or membrane bound bodies.

Composition of Hemosiderin

In contrast to the water soluble and highly structured ferritin, hemosiderin is characterized by its water insolubility and heterogeneity. It has been generally accepted that hemosiderin has a higher iron to organics ratio than ferritin with evidence of lipid, carbohydrate, sialic acid and porphyrin in some preparations [112–115]. The particle size of hemosiderin varies considerably [93,111,116–118]. In addition, the mineral component of hemosiderin is not spatially constrained by its organic component in contrast to ferritin where the maximum particle size of the mineral is limited to the size of the protein cavity. This observation suggests that the iron oxide component in hemosiderin is formed by biologically induced (as opposed to biologically controlled) mineralization in which processes are not as tightly controlled by the organism as in the case of ferritin iron biomineralization. As such, the mineral particles typically have a larger size distribution and no unique morphology.

Chemical Speciation of Hemosiderin Iron

Most investigations of hemosiderin have been centred principally on the fact that hemosiderin has a high iron content. By chemical examination of hemosiderin granules in tissues, the form of iron present in hemosiderin was proposed to be iron(III) oxide or hydroxide impregnated in organic granules [61,64]. However, it is now known that hemosiderin exists in different forms based on different iron oxide mineral structures [119,120]. Significant differences have been observed in the properties of hemosiderin preparations based on their Mössbauer magnetic blocking temperatures and electron diffraction patterns. Hemosiderin iron(III) oxyhydroxide particles are now known to have three different structures: (a) based on ferrihydrite (similar to ferritin); (b) non-crystalline; and (c) based on the mineral goethite (α -FeOOH). Studies of the mineral structure of the iron(III) oxyhydroxide particles in hemosiderin have suggested that these different structures are formed in hemosiderin deposited under different biological conditions.

(a) Ferrihydrite like form

The most common form of hemosiderin is that based on the structure of the mineral ferrihydrite and is similar to the mineral cores of mammalian ferritins [59].

Electron diffraction patterns of this hemosiderin (and mammalian ferritins) show between two and six major rings with intensities and positions characteristic of ferrihydrite. Mössbauer spectra of these hemosiderins and ferritins show superparamagnetism with magnetic hyperfine splitting up to about 30 K for the poorly crystalline samples and up to 60 K for the well crystalline samples [59].

Hemosiderin with iron oxyhydroxide cores similar to the ferrihydrite cores of ferritin has been identified in normal human tissues [121], in the liver from an unphlebotomised genetic hemochromatosis patient, and in the livers of animals with either naturally occurring or artificially induced iron overload [120,122–127].

(b) Noncrystalline Iron Oxide Form

The second form of hemosiderin has no detectable medium or long range order of iron and oxygen atoms. Selected area electron diffraction experiments show no powder diffraction rings, presumably as a result of the absence of regular planes of atoms [120]. Mössbauer spectra show that magnetic hyperfine splitting is extinguished at about 4 K, indicating a very weak magnetic interaction between neighboring iron atoms [119]. The lack of order within the structure of this type of hemosiderin suggests that the iron is precipitated rapidly, that it is not a degradation product of ferritin and that it could be formed independently of ferritin. Such processes may occur when high levels of circulating nontransferrin bound iron are present. An amorphous iron(III) hydroxide hydrate is a product of the hydrolysis reaction of iron(III) which involves a complex sequence of reactions [103]. Initially, the formation of the low molecular weight species of mononuclear hydroxo complexes such as $\text{Fe}(\text{OH})_2^+$, $[\text{Fe}(\text{OH})]^{2+}$, $\text{Fe}_2(\text{OH})_2^{4+}$, $\text{Fe}(\text{OH})_4^-$ are formed [104]. These low molecular weight species interact to produce species with a higher nuclearity. Further polymerization processes produce amorphous solids. These solids are thermodynamically unstable and may gradually transform to more crystalline iron oxides or oxyhydroxides [104].

This noncrystalline iron(III) oxide form of hemosiderin has been found in the liver tissue of a patient with primary hemochromatosis who had undergone phlebotomy [119,120].

(c) Goethite Like Form

The third form of hemosiderin is more crystalline than the two described previously and may have a structure with aspects related to the mineral goethite. Goethite is the most common form of iron(III) oxyhydroxide in nature and is the polymorph to which most other FeOOH phases eventually transform upon ageing [103]. Goethite consists of double arrays of edge sharing $\text{FeO}_3(\text{OH})_3$ octahedra (Figure 5.5). The

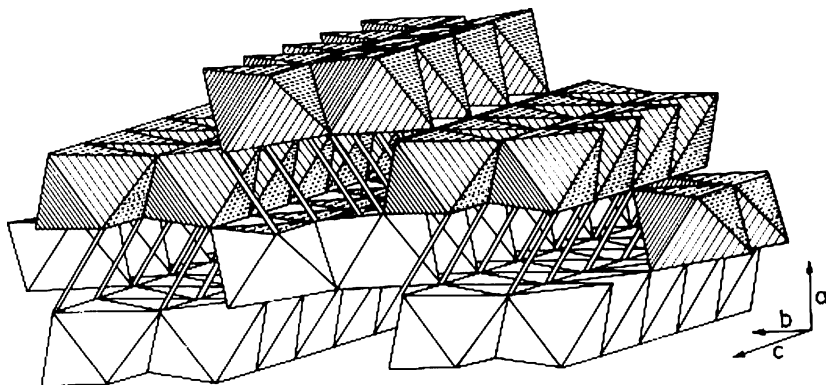


Figure 5.5 Diagram of goethite structure (reproduced from [128]).

double arrays of FeO_6 octahedra are linked by corner sharing in such a way as to form 2×1 octahedra ‘tunnels’ crossed by hydrogen bridges. The tunnels in goethite are only large enough to permit the passage of protons. In these FeOOH structures only half of the octahedral interstices are filled with iron(III) [128].

Using X-ray diffraction studies, a goethite like mineral form was reported in horse spleen hemosiderin [129,130]. Selected area electron diffraction patterns of the goethite like form of hemosiderin show rings with intensities and positions which indicate a defective goethite like structure [120]. Mössbauer spectra of goethite like hemosiderins have shown magnetic hyperfine splitting at temperatures in excess of 78 K. These higher magnetic blocking temperatures reflect the larger magnetic anisotropy energies of these materials compared with the ferrihydrite based hemosiderins [59,119].

The significance of the differences between the different forms of iron oxyhydroxide was recognised by Rimbert *et al.* [131]. They reported differences in the Mössbauer spectra of the livers of rats which had been loaded by carbonyl iron dietary supplementation and of β -thalassemia patients who had regular blood transfusions. Iron stored in the β -thalassemic liver showed typical high spin iron(III) with superparamagnetic behaviour. The Mössbauer spectra consisted of a doublet with quadrupole splitting smaller than that of ferritin together with a sextet which persisted to temperatures above 78 K. The magnetic hyperfine field splitting in the rat liver samples was extinguished below 78 K. Rimbert *et al.* [131] suggested that the difference between the two types of iron deposit was due to the physiological nature of the iron overload. Later, with the combination of Mössbauer spectroscopy, electron diffraction and electron microscopy, the form of iron oxyhydroxide contributing to the magnetic hyperfine field splitting above 78 K was identified as the goethite like iron ‘cores’ of hemosiderin [119,120]. This type of hemosiderin has also been found in the livers and spleens of other patients with secondary iron overload owing to the treatment of thalassemia by repeated blood transfusions [117,121,132]. In addition, no goethite like form of hemosiderin has been found in normal human liver and spleen [117,121,133]. The presence of the goethite like form of hemosiderin in a primary hemochromatotic patient has been reported [126]. In addition, this form of hemosiderin has been reported in a liver without pathological damage, namely, from the sea mammal *Dugong dugon* [134].

5.1.5 DISEASE SPECIFIC CHEMICAL SPECIATION OF HEMOSIDERIN IRON

Evidence for a relationship between the form of hemosiderin iron deposited in tissues and the disease state of the subject has been reported in studies of human spleen tissue from two identifiably different groups of patients [135]. Mössbauer spectra of 12 β -thalassemia/hemoglobin E spleen samples from Thai patients who

had not received multiple blood transfusions and chelation therapy and 7 β -thalassemia spleen samples from Australian patients who had received multiple blood transfusions and chelation therapy were recorded with sample temperatures of 78 K. Each spectrum was found to consist of a superposition of a relatively intense central doublet characteristic of high spin Fe(III), a low intensity sextet of peaks owing to magnetic hyperfine field splitting, and occasionally a doublet that could be attributed to heme iron. A significant ($p = 0.01$) difference (Kolmogorov–Smirnov statistic of 0.71) between the distribution of sextet signal intensity as a fraction (F_s) of the total nonheme iron Mössbauer spectral signal for the two groups of patients was detected. The distribution of F_s for the Thai β -thalassemia/hemoglobin E spleens had a mean value of 0.128 (s.d. 0.035) while that for the Australian β -thalassemia spleens had a mean of 0.27 (s.d. 0.12). No significant difference in the distribution of nonheme iron concentrations in the tissues between the two groups of patients was detected by atomic absorption spectrometry. This study showed that the Australian β -thalassemia patients had a higher fraction of their nonheme spleen iron in a goethite-like form than the Thai β -thalassemia/Hb E patients.

There could be several reasons for the difference in chemical speciation of spleen iron between the two groups of thalassemia patients studied including: (a) the different genotypes of the two groups; (b) the effect of blood transfusions; (c) the effect of chelation therapy; (d) differences in diet. The fact that there is different chemical speciation of iron in tissues between different identifiable groups of patients has implications for the treatment and management of iron overload diseases. The reactivities of the different iron oxide forms are expected to be different (see later).

5.1.6 TRANSFORMATION OF FERRIHYDRITE TO GOETHITE

The major structural differences between the goethite like form of hemosiderin and the ferrihydrite core of ferritin suggests that it may be formed independently from ferritin. However, one possibility to be considered is the transformation of ferrihydrite to a more stable form. Naturally occurring ferrihydrite can be transformed to other phases of iron oxides, particularly hematite, goethite or a mixture of the two [136]. These compounds form by different and competitive pathways [136,137]. The conditions favourable for the formation of goethite are unfavourable for the formation of haematite and vice versa. The master variable governing the rates at which these compounds form is pH. Other important factors are temperature and the presence of additives [104]. Most additives retard the transformation and by suppressing formation of α -FeOOH, lead to an increase in the amount of α -Fe₂O₃ in the product. However, some additives also directly promote formation of the latter compound.

Formation of hematite involves an internal dehydration and rearrangement process within the particle of ferrihydrite [138]. Any factors which induce aggregation of ferrihydrite particles (pH near the point of zero charge (pzc) of

ferrihydrite, pH 7–8) or dehydration (i.e. increased temperature) would promote the formation of hematite [104,137].

The formation of goethite proceeds by a different pathway, namely dissolution of the ferrihydrite and reprecipitation of the iron [104]. The reaction rate is governed by the rate of dissolution of ferrihydrite or by the rate of nucleation/crystal growth of goethite. Formation of goethite is favoured by raising or lowering the pH away from the pzc, thus promoting dissolution of ferrihydrite [136].

In addition, the rate of goethite crystallization is significantly influenced by both the temperature and the initial solution pH. The morphology of the goethite formed is also a function of the pH of the system [139]. The synthesis of goethite in highly alkaline solutions causes the precipitate to form as acicular needles with sizes often ranging between 0.1 mm to several millimeters [140]. According to a more refined model of this two step reconstructive transformation, goethite is formed via solution, preferably from monovalent iron(III) ions $[\text{Fe}(\text{OH})]^{2+}$ and $\text{Fe}(\text{OH})_4^-$ [136]. In highly alkaline aqueous solution, $\text{Fe}(\text{OH})_4^-$ is the dominant dissolved species, whereas $[\text{Fe}(\text{OH})]^{2+}$ dominates in the neutral pH range [141].

Goethite also can be produced in the range of pH 7–8 as small, almost isotropic crystals with a narrow size distribution [140]. In this system, an organic ligand such as cysteine is used as a reductant in order to dissolve the ferrihydrite. In the first stage of dissolution, amorphous iron(III) hydroxide (ferrihydrite) is converted to a more crystalline iron(II)/iron(III) intermediate by interaction with a reducing organic ligand. This intermediate phase is then oxidized to poorly ordered $\alpha\text{-FeOOH}$. The transformation to further crystalline material involves precipitation of dissolved iron(III) species onto the initial very poorly ordered crystal.

Evidence suggests that the different forms of iron oxide in hemosiderin are determined by the prevailing biological/pathological conditions and may reflect diverse mechanisms of formation. For example, the presence of the ferrihydrite form of hemosiderin suggests, but does not confirm, that it is derived from ferritin. These findings lead to various questions regarding the mechanisms of formation, biochemistry and magnetic properties of different forms of iron oxide in hemosiderin.

5.2 REACTIVITY OF PATHOLOGICAL IRON OXIDE DEPOSITS

5.2.1 IRON OXIDE ISOLATION AND CHARACTERIZATION

Iron storage proteins can be isolated from tissues by various means, depending on their properties. Ferritin can be isolated from tissues on the basis of its characteristic behaviour of water solubility and heat resistance up to 70 °C. The insolubility of hemosiderin allows this material to be isolated but also is a major factor limiting its purification. Several extraction methods have been described, based on the insolubility of hemosiderin in water and the density of its granules [63,112,130,142–146]. Some modifications to these extraction processes, such as

magnetic separation [144], mechanical separation from the frozen sediment [130] or differential centrifugation in high density media [112,145] have been developed to increase the yield and 'purity' of the isolated hemosiderin.

The different results obtained in previous studies of the chemical analysis of extracted hemosiderin [124,126] can be attributed to the difficulty of obtaining 'pure' hemosiderin uncontaminated by other cellular components. However, its heterogenous nature makes these results difficult to assess. Hemosiderin preparations tend to have higher iron to organics ratios than ferritin with evidence of lipid, carbohydrate and porphyrin in some of the preparations [60,61,114,115,143,145,146].

Mössbauer spectroscopic studies of hemosiderin extracted from the spleens of thalassemic patients have shown considerable differences from the corresponding ferritin with regard to their superparamagnetic behaviour [117]. This hemosiderin contains the goethite like form of iron(III) oxyhydroxide, with a more crystalline structure than the ferrihydrite found in ferritin. The study by St Pierre *et al.* [135] on the chemical speciation of hemosiderin iron in two identifiably different groups of thalassemia subjects indicated that the fraction of goethite like form of hemosiderin in the tissues may depend on factors such as the medical treatment received by the patients (e.g. chelation therapy and blood transfusion), the type of disease or genetics.

In order to study the hemosiderin in tissues with physical techniques sensitive to iron and/or crystalline material (e.g. Mössbauer spectroscopy, TEM, ED), the concept of 'crude hemosiderin' has been introduced to describe the water insoluble fraction remaining after the water soluble fraction has been removed from homogenised tissues [147]. The removal of the water soluble component should, in principle, remove all water soluble iron proteins, including ferritin, hemoglobin, myoglobin, cytochrome c, etc. Thus, experiments designed to study the iron in the sample (e.g. Mössbauer spectroscopy) or electron dense particles (TEM, ED) should give information on the hemosiderin iron only. The advantage of the 'crude hemosiderin' concept is that all of the insoluble fraction is studied. More highly purified hemosiderins are known to lose certain components during the purification procedures [126,147].

Human tissues used for crude hemosiderin preparation were obtained from thalassemia patients who had undergone minimal blood transfusion without chelation therapy and from patients who had undergone regular blood transfusion with chelation therapy. The range of the tissue samples had varied iron contents. Crude hemosiderin was prepared from those tissues displaying a representative range of iron content. Density centrifugation was used in some cases to further purify the hemosiderin. Mössbauer spectroscopy was used to characterize the hemosiderin particularly to determine the presence of the goethite like form of iron oxide. The morphology and particle size of the isolated ferritin and hemosiderin were studied using TEM. Other factors evaluated were the variation in iron content of hemosiderin, the phosphate to iron ratio and the variation in organic components.

5.2.2 MATERIALS

The human tissues (liver and spleen) used for the isolation of ferritin and crude hemosiderin were obtained either post mortem or were removed after splenectomy of β -thalassemic/Hb E patients (Thai patients) and β -thalassemic patients (Australian patients) (see St Pierre *et al.* [135]). As mentioned previously, β -thalassemic patients in Australia are regularly transfused with packed red cells and receive regular chelation therapy while standard care for β -thalassemic/Hb E patients in Thailand does not involve regular transfusions or chelation therapy. Hence the ferrokinetics in these two different groups of patients are likely to be very different.

Four livers and spleens from Thai β -thalassemic/Hb E tissues and four spleens from Australian β -thalassemic tissue were selected for the ferritin and hemosiderin isolations. The ferritin extracted from β -thalassemic/Hb E livers and spleens was pooled so that a single sample each of β -thalassemic/Hb E liver and spleen ferritin was represented. One ferritin isolated from a spleen obtained by splenectomy from a β -thalassemic patient who had received regular blood transfusion and chelation therapy was studied separately. Dugong liver tissue was also included as a comparison material, since this sample has been reported to have very high iron content and fraction of goethite like hemosiderin [134]. The synthetic iron oxides (ferrihydrite and goethite) were included as control materials which, furthermore, do not contain any organic components.

5.2.3 ISOLATION OF THE IRON STORAGE COMPONENTS FROM TISSUES

Hemosiderin

Each tissue was homogenized at 4°C in sodium chloride solution (0.9 % w/v NaCl, AR, Unilab®) in a ratio of 1 g tissue to 5 ml of the solution, using a Polytron® homogenizer (Kinematica GmbH, Luzern, Switzerland). Protease inhibitor, 0.12 mM phenylmethylsulfonyl fluoride (PMSF, AR, Sigma®) in propan-1-ol (AR, Sigma®), was added to the NaCl solution. The homogenates were centrifuged at 6080 g for 30 min at 4°C in a Sorvall centrifuge (SS/1 Sorvall). The supernatant solutions were retained and the pellet was rehomogenized in the NaCl solution before being centrifuged again 2–3 more times. The supernatants were pooled ready for further ferritin purification. The pellet was washed by centrifugation with the NaCl solution until a clear supernatant was obtained. In the final step, this pellet was washed again with distilled water. This fraction was classified as ‘crude hemosiderin’.

Crude hemosiderin from some samples was further purified by using the potassium iodide (KI, AR, BDH®) density centrifugation method [112,145]. Each crude hemosiderin sample was centrifuged twice for 10 min each at 12 500 rpm, 4°C with KI solution (0.4 M). The final precipitate was collected and copiously washed three times with double distilled water. The final precipitate was frozen and

freeze dried, then ground to a fine powder. The samples were kept in a desiccator for further study.

In a further purification, a sample of crude liver hemosiderin was treated by magnetic separation and chemical extraction. The magnetic separation was carried out using modification of a previously reported method [144]. Crude hemosiderin suspended in a high density NaCl medium was passed through a magnetic gradient of a permanent magnet (NdFeB) mounted outside a perspex cell separator. The selected hemosiderin fraction that was attached to the magnet was then washed with deionized water before being freeze dried.

A second sample of the crude liver hemosiderin was photochemically and chemically treated in order to eliminate the organic material of hemosiderin while limiting any change in the form of iron oxide. Dry crude hemosiderin in a quartz tube was exposed to ultraviolet (UV) radiation for 200 hours. The system was water cooled to prevent thermal transformation of the iron oxide. The radiated energy in UV range was about 84 Watts from an ACE–Hanovia pressure quartz 450 mercury vapour lamp. After UV radiation, a fraction of the dry hemosiderin was sonicated in dimethyl sulphoxide (DMSO, AR, Unilab®) for three 5 min intervals before the organic solvent was discarded. The samples were then kept under vacuum before examination by infrared spectroscopy.

Ferritin Purification

To purify ferritin, the pooled supernatant from the hemosiderin extraction was heated in a waterbath at 70 – 75°C for 10 min, immediately cooled in an ice bath and then centrifuged at 6080 g (4°C) for 30 min. The pellets of denatured organic material were discarded and the supernatant was collected and filtered by gravity filtration using Whatman no. 1 filter paper. Sodium azide (AR, Sigma®) was added to the supernatant solution to give a final concentration of 0.02 % (w/v). Ultrafiltration using a Diaflo® PM30 membrane (exclusion limit of 3×10^4 Daltons, Amicon Corporation, Lexington USA) under a nitrogen pressure of 4.0 atm at 4°C concentrated the ferritin solution prior to chromatography. At the same time, the solvent was also changed to borate buffer (AR, BDH®; 0.025 M borate, 0.15 M NaCl, pH 8.6).

Ferritin samples were further purified by column chromatography using Sephadex G-75 followed by Sephacryl-300 (Pharmacia Fine Chemicals) in borate buffer pH 8.6 as the mobile phase. The flow rate of the mobile phase was adjusted by a peristaltic pump (P-3, Pharmacia Fine Chemicals). Fractions (2 ml) were collected by an automatic fraction collector (Frac-160, Pharmacia Fine Chemicals). The peak corresponding to ferritin was identified by using a combination of UV-visible spectrophotometry (Hewlett-Packard 8450 A) at 280 nm, for protein and qualitative measurement for iron using potassium ferrocyanide (2 % w/v, AR, Mallinckrodt®) and hydrochloric acid (2 % v/v, AR, Univar®). Finally, the ferritin was concentrated by ultrafiltration with a Diaflo® PM30 membrane. The borate buffer was replaced by Millipore® water prior to further fast freezing and freeze drying for storage until use.

Synthesis of Iron Oxides From Iron(III) Systems

The synthetic ferrihydrite and goethite control material were prepared following the procedures of Schwertman and Cornell [128]. Crystalline ferrihydrite was prepared by rapid hydrolysis of a 1 % (w/v) iron(III) solution. The solution was prepared by adding $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ (AR, Univar®) to preheated distilled water at 75 °C with rapid stirring. The mixture was kept at 75 °C for 10 min before cooling rapidly in an ice bath. The solution was then subjected to ultrafiltration with a Diaflo® PM30 membrane. The ferrihydrite solution was then dialyzed against water. The water was changed 3–4 times during 3 days of dialysis. The suspension was then freeze dried and powdered.

Goethite was prepared by holding freshly precipitated ferrihydrite under alkaline conditions for several days. Under these conditions, the precipitate dissolved to release the soluble iron(III) species $\text{Fe}(\text{OH})_4^-$ from which the less soluble goethite nucleates and grows. Poorly ordered ferrihydrite was first prepared by mixing a freshly prepared 1 M $\text{Fe}(\text{NO}_3)_3$ (AR, Univar®) with 5 M KOH (AR, Univar®) solution in the ratio of 1:1.8. The less crystalline ferrihydrite precipitates at this stage. The mixture was then diluted with distilled water to ten times the original volume in a closed polyethylene container. This solution was kept in an oven at 70 °C for 60 hours. The yellow-brown precipitate was centrifuged and washed three times (5000 rpm, 20 min) with distilled water and freeze dried. The sample was then ground to obtain a uniform grain size.

The glassware used in this study was acid washed, and all chemicals were analytical reagent (AR) grade.

5.2.4 METHODS

Elemental Analysis and Mössbauer Spectroscopy

The fine, powdered freeze dried samples were acid digested for analysis for elemental components (iron and phosphorus) using an inductively coupled plasma atomic emission spectrometer (ICP; Varian Liberty 200). Samples containing approximately 2 mg of iron each were used for Mössbauer spectroscopy.

Transmission Electron Microscopy and Electron Diffraction

Diluted ferritins, solubilized crude hemosiderin in tetramethyl ammonium hydroxide (TAH, AR, Sigma®) [112], and ultrasonically dispersed iron oxide preparations were air dried onto Formvar films supported by 200 mesh copper grids. Electron diffraction was performed using a transmission electron microscope (Jeol 2000). Unsolubilized crude hemosiderin was also processed into ultrathin sections for electron microscopy.

Iron Oxyhydroxide Particle Size Measurement

Ferritin and solubilized crude hemosiderin were used for this determination. The ferritin samples included pooled liver ferritin, pooled spleen ferritin and spleen ferritin from the β -thalassemic patient (Australian) who had received regular blood transfusions and desferrioxamine (DFO) treatment. For crude hemosiderin, there were samples of β -thalassemic/Hb E spleen hemosiderin (Thai samples) and β -thalassemic spleen hemosiderins (two Australian samples).

The electron dense particles of the isolated samples of solubilized hemosiderin and ferritin were measured from enlarged electron micrographs. For each sample, a grid was placed over the micrograph and cores were measured in sequence by scanning across each row of the grid in turn until 150 cores had been measured. Catalase crystals were used to calibrate the magnification. The electron micrographs of the catalase crystal and the isolated iron oxides were taken at the same magnification. The largest dimension of the irregular electron dense particles was measured. The core sizes were measured in millimetres to two decimal places using vernier callipers and the measurements converted to nanometers.

Infrared Spectroscopy

Infrared spectra of the samples of isolated ferritin and hemosiderin, as well as the synthetic ferrihydrite and goethite, were measured over the wavelength range of $4000\text{--}400\text{ cm}^{-1}$ on a Nicolet® Magna infrared spectrometer (System 850). The spectrometer was operated with a diffuse reflectance unit. This method of sample examination uses a mixture of sample and KBr. The mixture was ground as finely as possible before placing into a 3 mm diameter microsampling cup. This method has the advantage of lower water contamination because of the ease of sample preparation compared with the KBr disc method. The blank KBr spectrum provided the background for subtraction. Sample spectra were baseline corrected and smoothed using the Nicolet instrumental software (OMNIC®) provided.

5.2.5 RESULTS

A relatively high percentage of the Mössbauer spectral area at 78 K (tissue, $41 \pm 12\%$; hemosiderin, $60 \pm 8\%$) is given by the sextet component in the spectra of whole spleen tissue and of hemosiderin isolated from the treated β -thalassemic patients who have undergone blood transfusion and chelation therapy. This can be seen in Figures 5.6a and 5.6b. The Mössbauer spectra at 78 K of the isolated crude hemosiderins showed that the percentage area of the sextet varied between 8% and 60%. No significant sextet was observed for any of the ferritin samples, as illustrated in Figure 5.6c.

These typical Mössbauer spectra at 78 K shown in Figure 5.6 were recorded on samples quite low in iron concentration thus giving comparatively low signal to

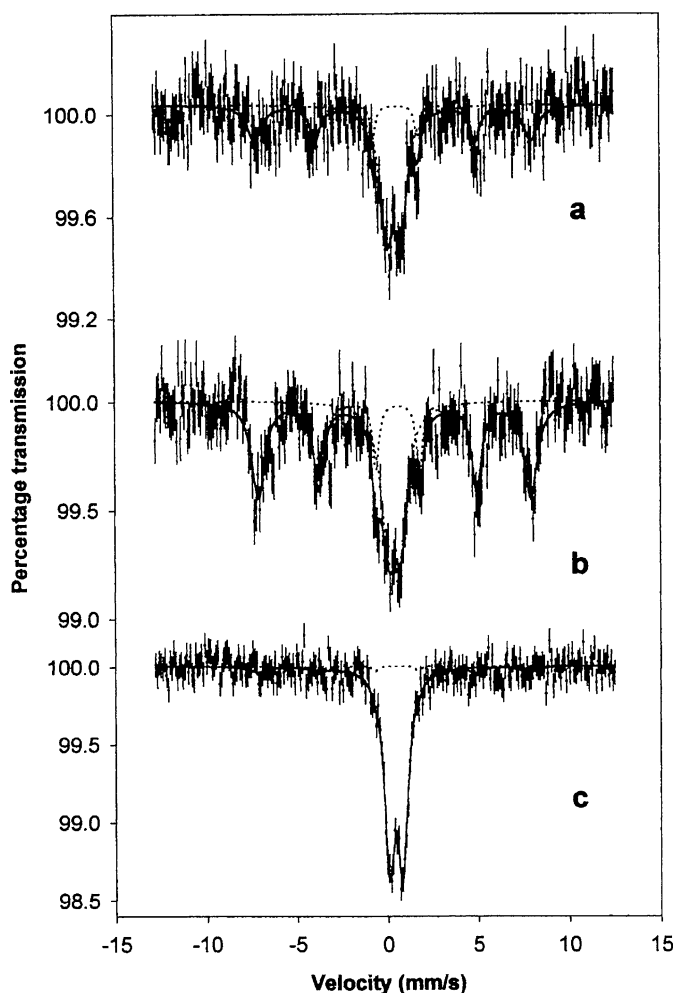


Figure 5.6 Mössbauer spectra of (a) β -thalassemic spleen whole tissue, and (b) its crude hemosiderin and (c) purified ferritin, both isolated from this tissue.

noise ratios. Figure 5.7 illustrates the TEM image of this spleen tissue which has a large fraction of its hemosiderin in goethite like form. The clumps of electron dense material are located mainly intracellularly (Figure 5.7a) and are composed of aggregates of electron dense particles (Figure 5.7b). The ferritin fraction from the same tissue appeared as discrete, electron dense particles (Figure 5.7c). The ultrathin section of isolated crude hemosiderin from this same tissue showed clumps of electron dense material similar to those seen in the spleen tissue, packed within limited bodies. These bodies were contained or embedded within other membranous complexes (Figure 5.7d). At higher magnification (Figure 5.7e), these electron

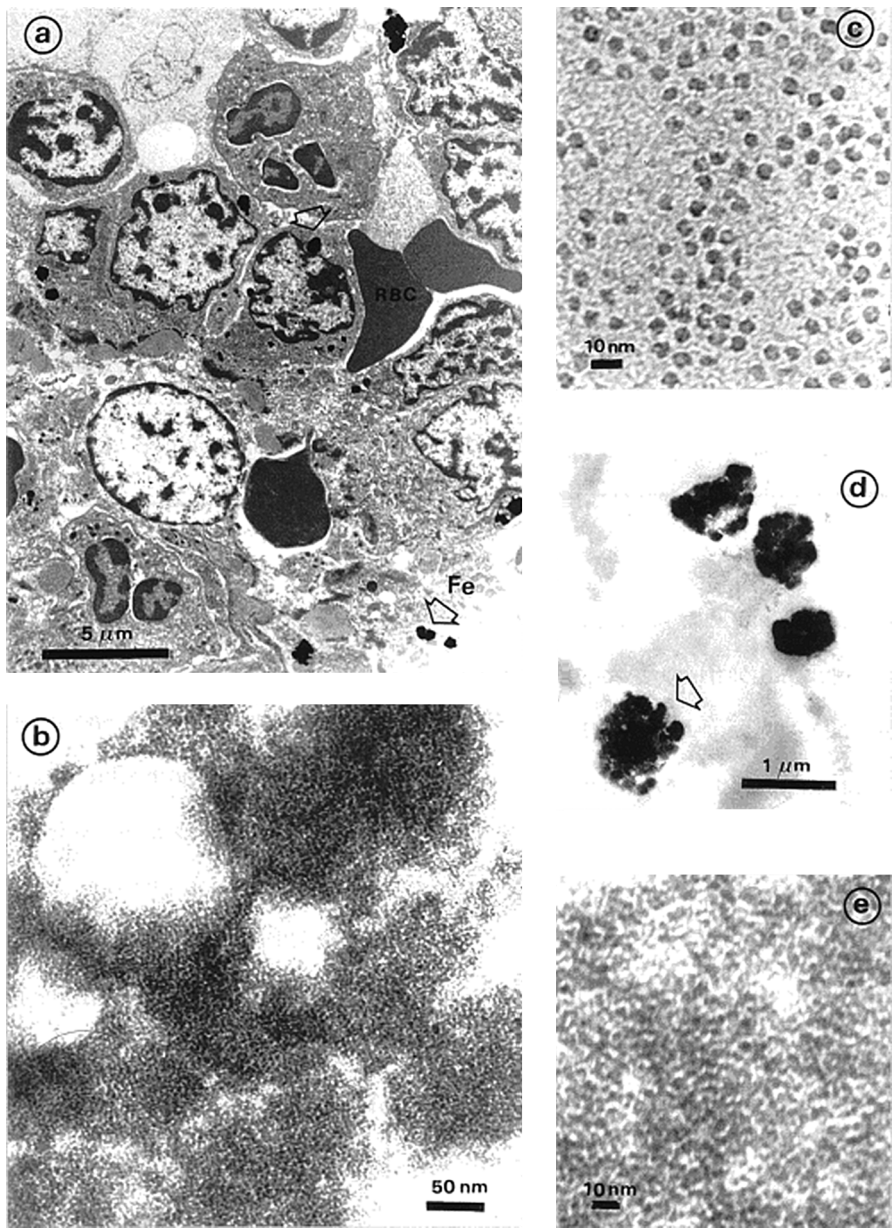


Figure 5.7 Transmission electron micrographs of (a) a β -thalassaemic spleen, (b) tissue iron deposit at high magnification. Isolated ferritin (c) and the thin section of isolated crude hemosiderin of this tissue are shown in (d) and at a higher magnification (e).

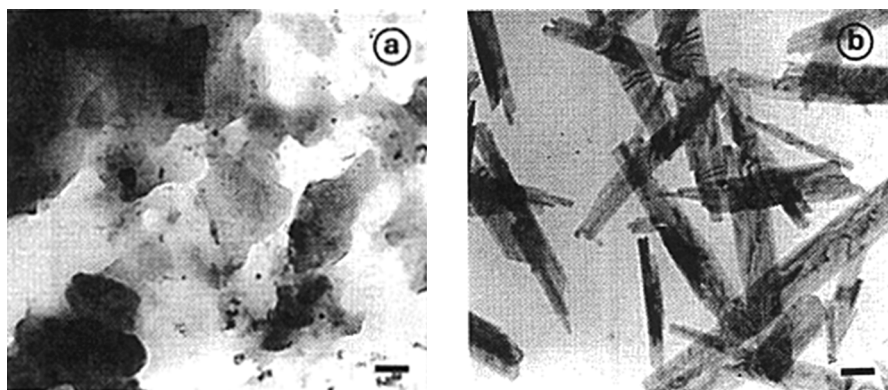


Figure 5.8 Synthetic iron oxides: (a) ferrihydrite, (b) goethite (scale bar = 100 nm).

dense hemosiderin particles also showed aggregated and closely packed electron dense iron cores. The morphology of the iron oxides isolated from tissues is quite different from that seen in the synthetic iron oxides (Figure 5.8). The synthetic goethite formed highly crystalline elongated acicular crystals, while at the same magnification, the synthetic ferrihydrite showed no crystal like feature.

Selected area electron diffraction from both the Australian β -thalassemic spleen ferritin and the hemosiderin isolated from the same tissue gave powder diffraction rings from at least one of the selected areas on the grid. Table 5.1 shows d-spacings calculated from the reflections. Relative intensities of the reflections, have not been included in the table because different exposure times were used for different reflections thus making intensity comparisons difficult. All of the reflections were of relatively low intensity and were rather diffuse. However, both specimens gave reflections that could be identified with the reflections expected for the mineral ferrihydrite (Table 5.1). Both of the specimens also gave several extra reflections at higher angles than those covering the range previously reported for ferrihydrite [148] (corresponding to d-spacings $\leq 1 \text{ \AA}$) as previously reported for ferritin cores [86,118]. However, the hemosiderin sample gave reflections at angles lower than those covering the range of ferrihydrite reflections, corresponding to d-spacings of $2.73 \text{ \AA}$ and $3.55 \text{ \AA}$. The d-spacing of $2.73 \text{ \AA}$ is very close to those measured in hemosiderin isolated from the livers and spleens of three multiply transfused β -thalassemic patients [120] (Table 5.1). However, the d-spacing of $3.55 \text{ \AA}$ has not been observed previously in any other tissue iron oxide deposits.

Isolation of Hemosiderin

Crude hemosiderin from a β -thalassemic/Hb E liver and the dugong liver were purified further using magnetic isolation and KI centrifugation. The series of IR spectra shown in Figure 5.9 include those from a crude liver hemosiderin

Table 5.1 Electron diffraction data from tissue iron oxide deposits and related materials.

Specimens		d-spacing (Å)													
(a)	Aus thal Fn		2.53	2.24	1.97	1.72	1.53	1.47		1.29	1.12	1.04	0.88	0.85	0.82
(b)	Thai thal Fn		2.51	2.25	1.99	1.74		1.47	1.34				0.87		
(c)	Fn crystal		2.63	2.34	2.07	1.80	1.55	1.47			1.10	1.04	0.99	0.86	
(d)	Aus thal Hd	3.55	2.73	2.54	2.23	1.72	1.47	1.34			1.11				
(e)	Dugong liver		2.75	2.51	2.24	2.00	1.71	1.47	1.33		1.11	1.05	0.88		
(f)	Rat liver		2.51	2.23	1.82	1.71	1.47					1.06			
(g)	Ferrihydrite		2.56	2.23	1.98	1.73	1.55	1.47		1.18	1.11	1.05	0.91	0.88	0.81
(h)	Ferritin		2.5	2.23	1.99	1.72	1.50	1.47	1.33	1.23	1.18	1.05	0.91	0.88	0.84
(i)	Thal Fn		2.47	2.22	1.93	1.71		1.48							
(j)	Thal liver Hd	4.13	2.65	2.45	2.22	1.97	1.72	1.49	1.46						
(k)	Thal spleen Hd	4.25	2.68	2.45	2.20	1.99	1.71	1.54	1.47	1.43					
(l)	GH liver Hd		2.49		2.12		1.53								
(m)	Horse Hd		2.50	2.24	1.99	1.71	1.51	1.46							
(n)	Ferrihydrite		2.50	2.21	1.96	1.72	1.51	1.48							
(o)	Goethite	4.18	3.38	2.69	2.25	1.72	1.56	1.47*							

(a) Australian β -thalassemic spleen ferritin.

(b) Thai β -thalassemic/Hb E spleen ferritin.

(c) Horse spleen ferritin crystal fixed and embedded in resin.

(d) Australian β -thalassemic spleen hemosiderin.

(e) Dugong liver tissue section fixed and embedded in resin.

(f) Dietary iron loaded rat liver section fixed and embedded in resin.

(g) Ferrihydrite.

(h) Ferritin (source unspecified) [86,89].

(i) Thalassemic (transfused) ferritin from liver and spleen [120].

(j) Thalassemic (transfused) liver hemosiderin [120].

(k) Thalassemic (transfused) spleen hemosiderin [120].

(l) Genetic haemochromatosis liver hemosiderin [120].

(m) Horse spleen hemosiderin [123].

(n) Ferrihydrite [148].

(o) Goethite. Only intensities > 10 % of most intense peak shown.

*Reflection at $d = 1.47 \text{ \AA}$ is made up from three closely spaced weak reflections [148].

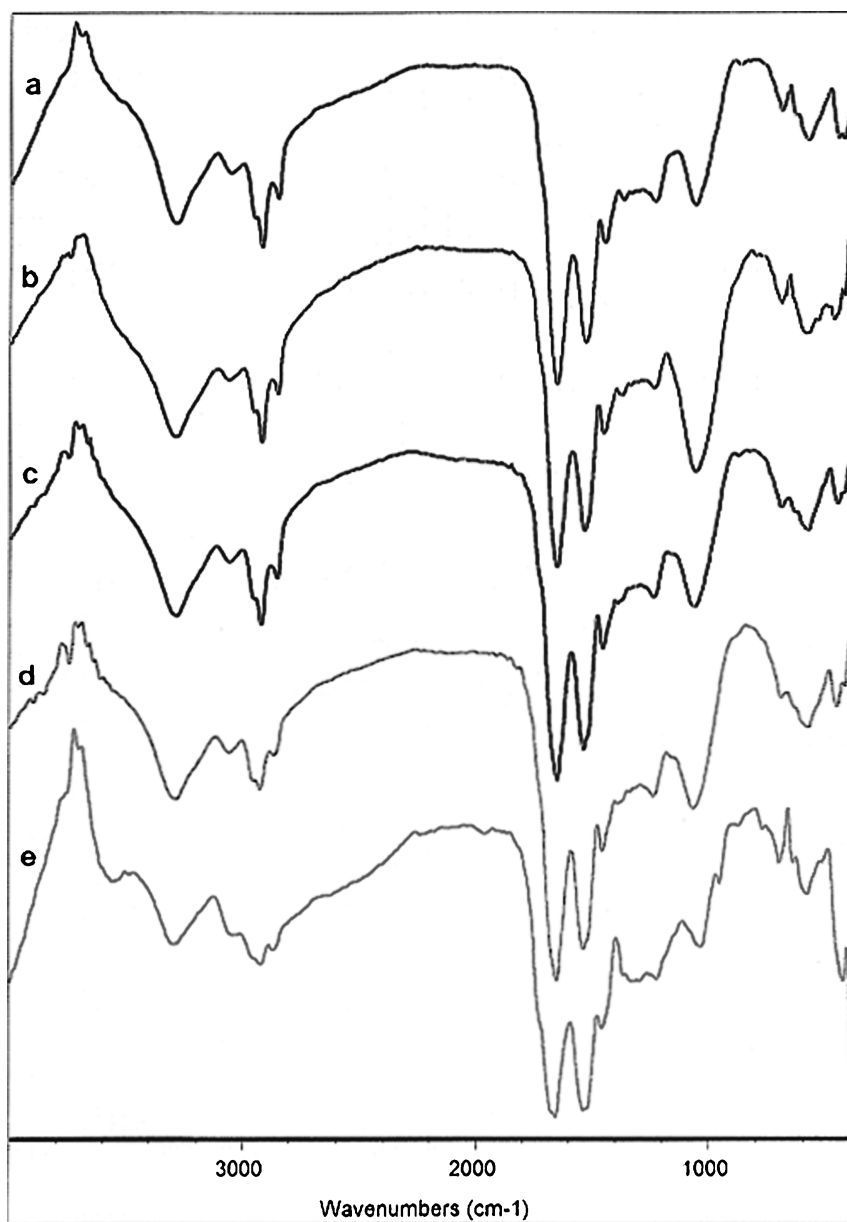


Figure 5.9 Infrared spectra of a β -thalassemic/Hb E liver hemosiderin prepared using various procedures: (a) crude, (b) KI centrifugation, (c) magnetic separation, the spectra of this crude hemosiderin following (d) UV treatment, (e) UV treatment and extraction with DMSO.

with various methods of isolation and treatment. Physical fractionation of crude hemosiderin by KI density centrifugation and magnetic separation does not significantly alter the resulting IR spectrum, although the concentrations of iron in the products of these isolation methods were slightly different from that of the original crude sample. The treatment with an organic solvent in combination with UV radiation has decreased the relative intensity of the peaks associated with the organic components. The ferrihydrite peaks at 680 cm^{-1} , 590 cm^{-1} and 450 cm^{-1} (Figure 5.14) and the additional band at 960 cm^{-1} were revealed in the sample after UV irradiation and treatment with DMFO.

The Concentration and Form of Iron in Tissues

There is a high degree of correlation between the iron concentrations in tissues and in their respective hemosiderins (both in the crude form and after KI isolation) (Figure 5.10a). In addition, the fraction of iron present as the goethite-like form of iron oxide in the crude hemosiderin is related to the fraction of iron present in this form in the tissue (Figure 5.10b). These relationships indicate that the concentration of iron and the form of iron present in any particular hemosiderin depends upon the concentration of iron and the form of iron in the whole of the tissue from which the hemosiderin was isolated.

The concentration of phosphorus in the samples of crude hemosiderin varied from 0.7 % to 2.3 % dry weight, without any significant correlation with the concentration of iron in either tissue or crude hemosiderin. The phosphorus to iron ratio (P:Fe) of the crude human hemosiderin varied between 0.2 and 18. Crude hemosiderin with high iron content tended to have low P:Fe ratios.

Iron Oxyhydroxide Particle Size of Crude Hemosiderin

The hemosiderin iron oxyhydroxide particle size measurements were performed using crude hemosiderin solubilized with tetramethylammonium hydroxide as previously reported [112]. Solubilized hemosiderins show electron dense particles under TEM similar to the cores observed *in situ* in the ultrathin unstained sections, except that they are more dispersed. The particle sizes of crude hemosiderins isolated from two Australian β -thalassemic spleens and a Thai thalassemic spleen (β -thalassemia/Hb E) were measured and are compared in Figure 5.11. These three crude hemosiderin samples have significant amounts of the goethite like form of hemosiderin component with F_s values of 0.10, 0.37 and 0.60 and average particle size distributions of $5.68\text{ (s.d. } \pm 1.80\text{)}$, $5.07\text{ (s.d. } \pm 1.89\text{)}$ and $4.17\text{ (s.d. } \pm 3.26\text{ nm)}$ respectively (Figures 5.11a, 5.11b and 5.11c). The TEM image of solubilized crude hemosiderins from the β -thalassemia/Hb E spleen (Figure 5.11a) displayed aggregation and the images of individual electron dense particles were difficult to resolve from one another. In comparison, the particles of hemosiderin from a

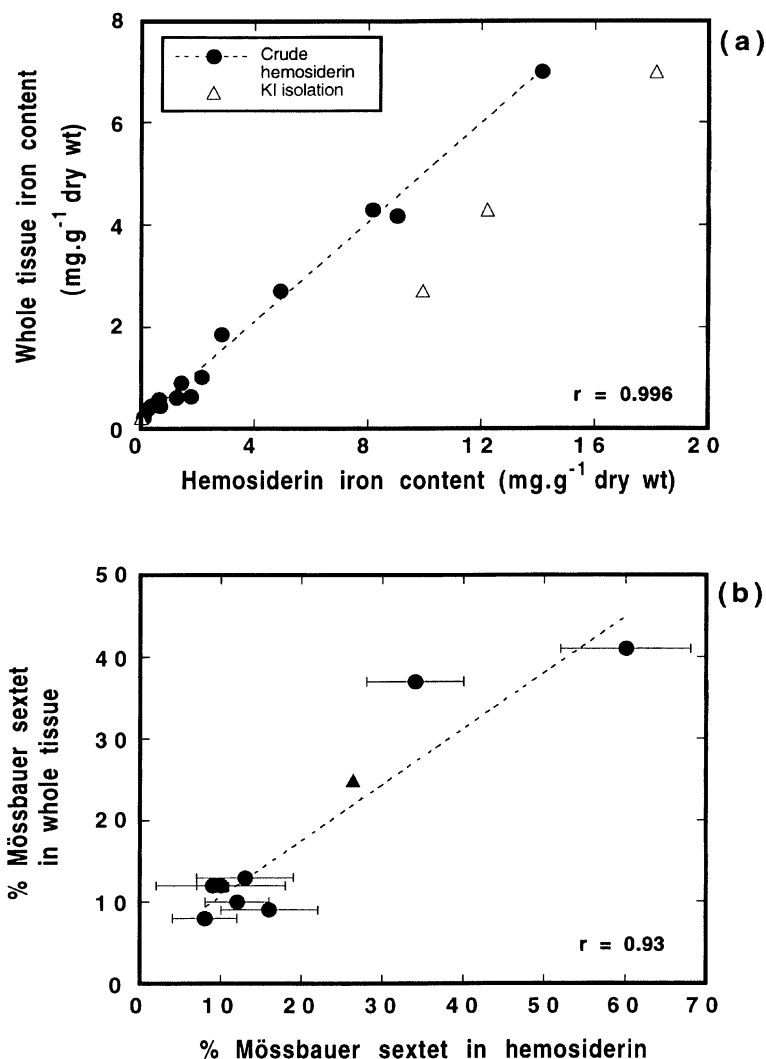


Figure 5.10 The correlation of (a) the concentrations of iron (%) in the whole tissues and their isolated crude hemosiderins, and (b) the percentage of sextet area from Mössbauer spectra of whole tissues and of their isolated crude hemosiderins (▲ is the value from [132]).

β -thalassemic spleen (which has $F_s = 0.60$) appeared discrete and electron dense with a wider range of particle sizes than that displayed by the other hemosiderin samples (Figure 5.11c). For this hemosiderin, there are three distinguishable populations of electron dense particles located in different areas of the examination grid. Separate micrographs of these three populations were taken and analyzed

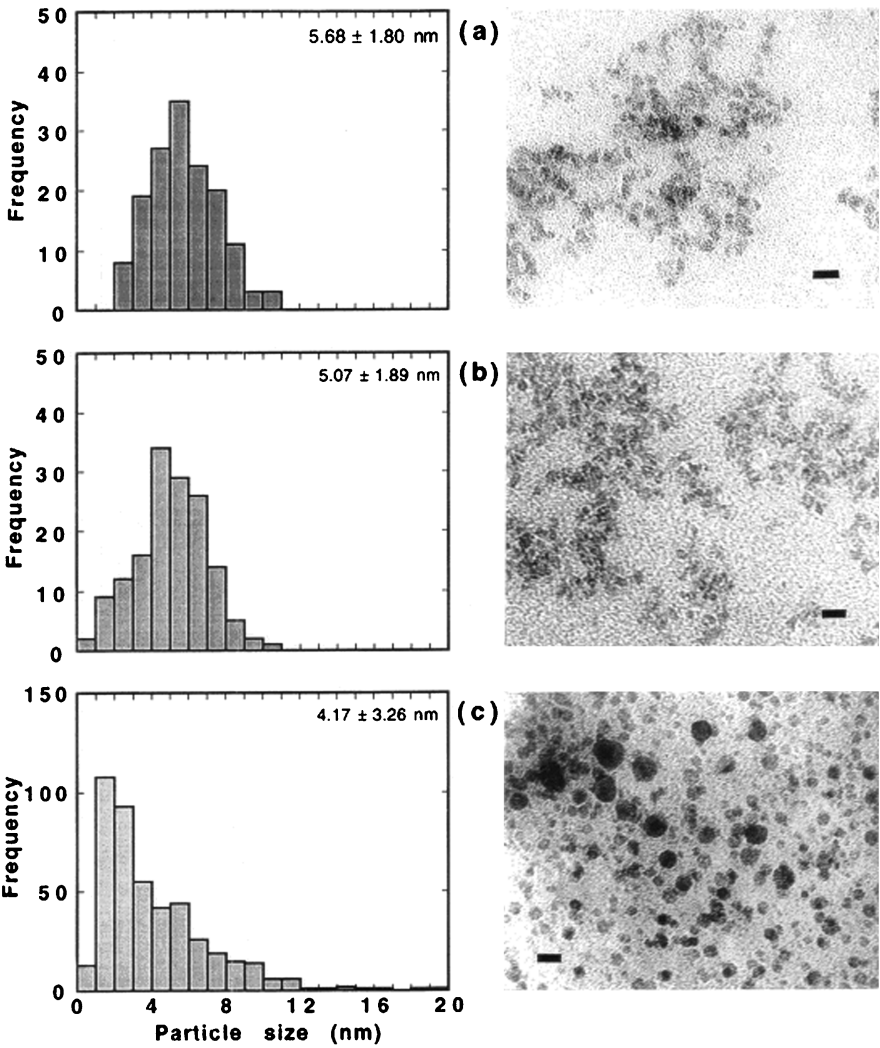


Figure 5.11 The particle size distribution of crude hemosiderin isolated from: (a) a β -thalassemic/Hb E spleen, (b) a β -thalassemic spleen, and (c) a β -thalassemic spleen with a high percentage of sextet. The appearance under the electron microscope is also shown (Scale bar is 10 nm).

for their size distributions. The size distribution graphs and images of these three populations are shown in Figure 5.12. The particle size distribution of these three populations were found to be 1.79 (s.d. $\pm$ 0.62), 3.96 (s.d. $\pm$ 1.80) and 6.76 (s.d. $\pm$ 3.98) nm (Figures 5.12a, 5.12b and 5.12c, respectively) giving an average value of 4.17 (s.d. $\pm$ 3.26) nm (Figure 5.11c).

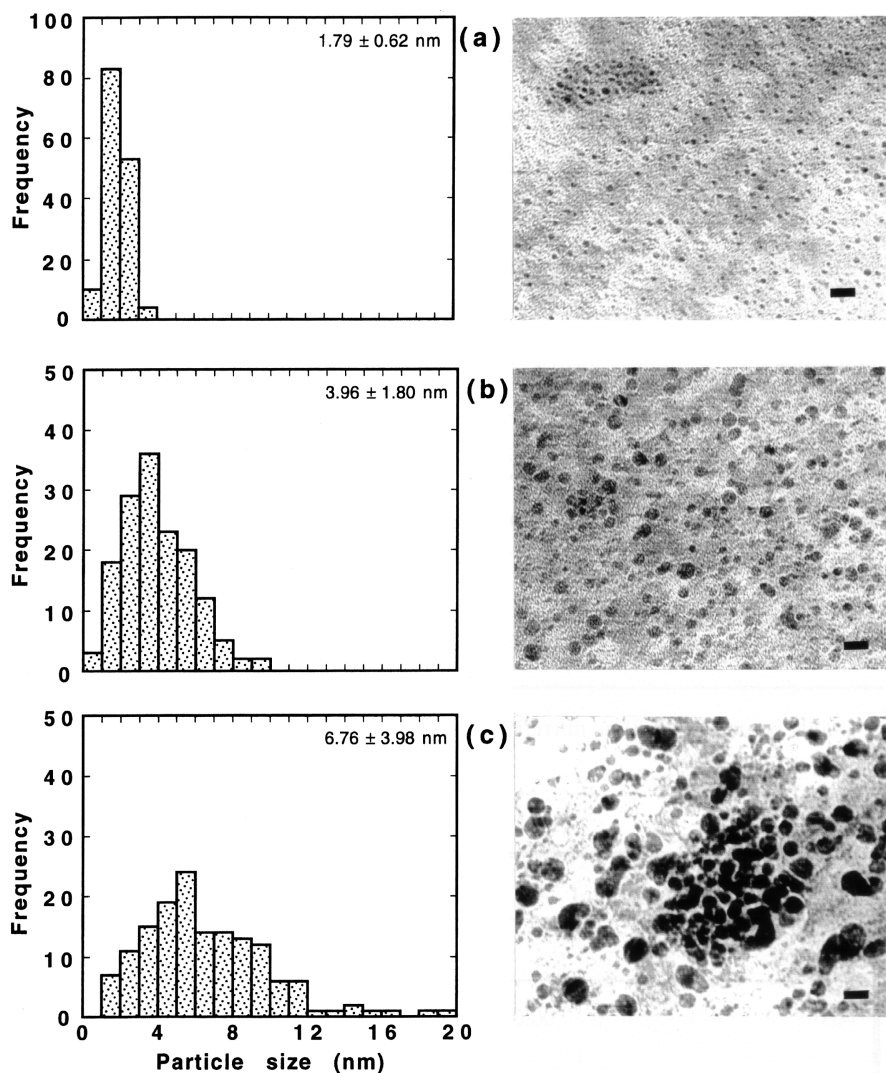


Figure 5.12 The particle size distribution of crude hemosiderin isolated from the β -thalassemic spleen with the high percentage of sextet in Mössbauer spectral signal, showing three distinctive size populations. The appearance under the electron microscope is also shown (scale bar is 10 nm).

Iron Oxyhydroxide Particle Size of Ferritins

The core size distribution of the purified ferritin isolated from an Australian β -thalassemic spleen was compared with that of a Thai β -thalassemic spleen ferritin sample (Figure 5.13). In the case of ferritin isolated from the Australian β -thalassemic spleen sample, the ferritin core size was 6.92 (s.d. ± 0.85) nm, significantly larger

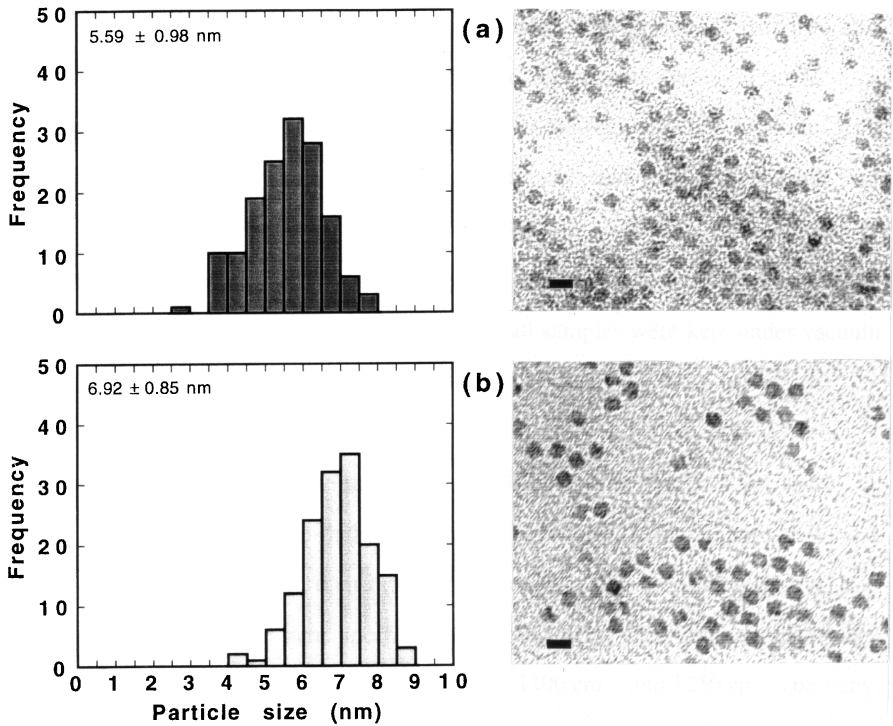


Figure 5.13 The particle size distribution of purified ferritin isolated from: (a) a Thai β -thalassemic/Hb E spleen compared with (b) the β -thalassemic spleen from a patient with DFO and blood transfusion treatment. The appearance under the electron microscope is also shown (scale bar is 10 nm).

than the β -thalassemia/Hb E spleen ferritin cores which were found to be 5.59 (s.d. ± 0.98) nm ($p < 0.001$). The TEM image of this β -thalassemic spleen ferritin had well defined cores with angular shapes. The P:Fe ratio of this β -thalassemic spleen ferritin was 0.07. In comparison, the P:Fe ratios of pooled liver and spleen ferritins isolated from β -thalassemic/Hb E tissues were much higher, 0.17 and 0.14 respectively.

Infrared Spectroscopy

Infrared (IR) spectra of hemosiderin and ferritin isolated from human livers and spleens were compared with those of the synthetic iron oxides (ferrihydrite and goethite) (Figure 5.14). There are obviously organic components present in the ferritins and crude hemosiderins, as shown by the peaks in the regions $3500\text{--}2000\text{ cm}^{-1}$ and $1700\text{--}1000\text{ cm}^{-1}$. These were not present in spectra obtained from the synthetic iron oxides (Figures 5.14a, 5.14b). The absorbances at 3400 cm^{-1} and 1640 cm^{-1} , due to OH stretching and bending respectively, are common features

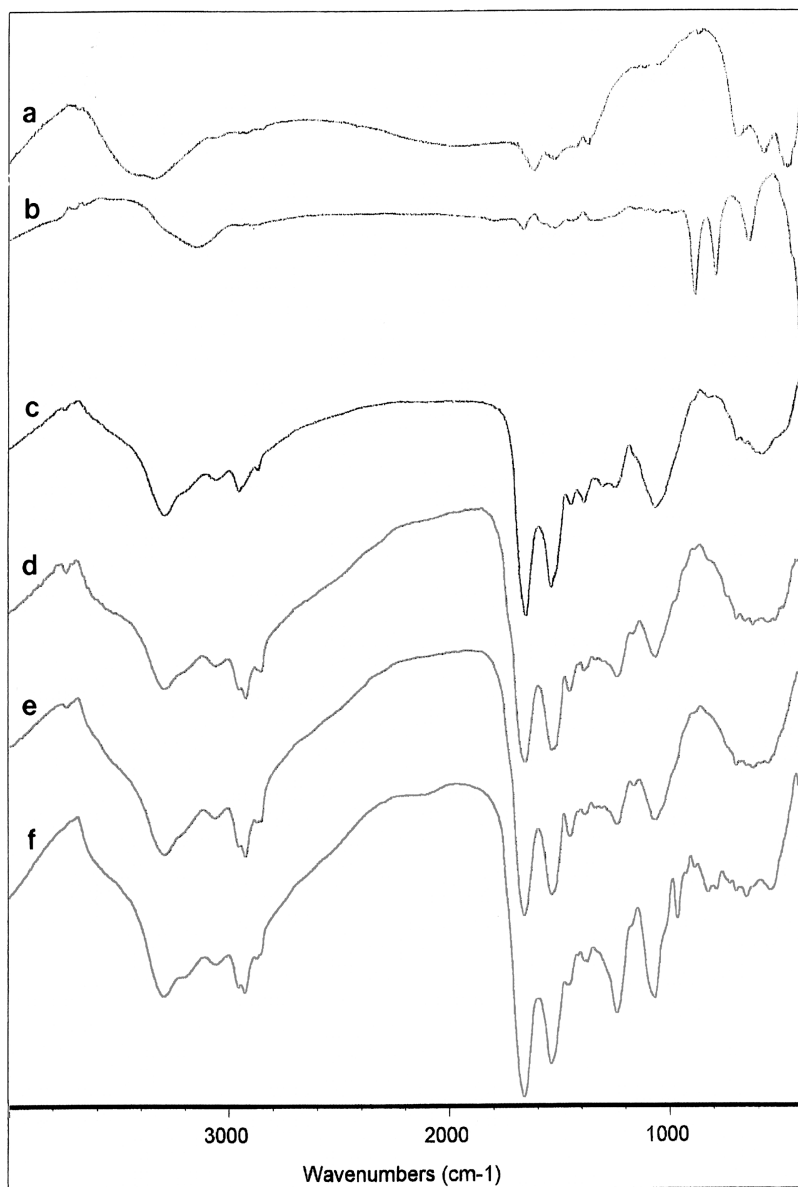


Figure 5.14 Infrared spectra comparing synthetic iron oxides, (a) ferrihydrite and (b) goethite, with the isolated iron oxides from the tissues: (c) β -thalassemic/Hb E spleen ferritin, (d) a β -thalassemic/Hb E liver hemosiderin, (e) a β -thalassemic/Hb E spleen hemosiderin, and (f) a β -thalassemic spleen hemosiderin.

indicating some molecular water and/or OH groups in all samples, although all samples were kept under vacuum prior to IR measurement.

In general, the spectra of human thalassemic spleen ferritin (Figure 5.14c) and crude hemosiderins isolated from β -thalassemia/Hb E liver and spleen (Figures 5.14d and 5.14e) show much similarity in their organic components. The ferritins from liver and spleen gave essentially the same spectra (result not shown). The inorganic phosphate absorption peaks are reported to occur at 960 cm^{-1} , 1100 cm^{-1} and 1250 cm^{-1} [149]. The intensity at 1250 cm^{-1} was less for ferritin than for hemosiderin samples. There was a strong absorption peak at 960 cm^{-1} in the hemosiderin sample from the β -thalassemic spleen (Figure 5.14f) and slight absorption in the other two hemosiderins (Figures 5.14d, and 5.14e). This peak is not seen in ferritin and synthetic iron oxide spectra.

Broad absorption bands in the low frequency region ($700\text{--}400\text{ cm}^{-1}$) were found in ferritin and hemosiderin spectra. However, in the crude β -thalassemic hemosiderin sample which had an F_s value of 0.60, well defined absorption peaks at 890 cm^{-1} , 795 cm^{-1} and 640 cm^{-1} were detected (Figure 5.14f). These peaks correspond to the absorption bands at 890 cm^{-1} , 796 cm^{-1} , 645 cm^{-1} , 406 cm^{-1} seen in the spectrum of the synthetic goethite (Figure 5.14b).

5.2.6 DISCUSSION

Hemosiderins isolated from different iron loading syndromes exhibit differences in both their peptide [124,126] and iron core composition [119,120,147]. The mix of various forms of iron oxide present in crude hemosiderin isolated from the thalassemic tissues (livers and spleens) in this study varies with the source of the samples. Mössbauer analysis of crude hemosiderin isolated from various tissues from patients with β -thalassemia/Hb E disease has shown a range of iron core sizes and variations in crystallinity together with three forms of iron oxide [147].

From the present study, the goethite like form of iron is still a major form of iron oxide found in the crude hemosiderin isolated from spleens of chelated and transfused β -thalassemic patients. Another study of hemosiderin isolated from a nonchelated patient with thalassemia showed that the goethite like form of iron oxide was the predominant form of iron oxide present ($F_s = 0.55$ and 0.47) [126]. One possible mechanism leading to the presence of different forms of iron oxide in hemosiderin is the removal of iron during treatment for iron overload [120,125,135,150]. These results, taken together, suggest that the goethite like form of hemosiderin present in the tissues may have many factors involved in its process of formation.

Electron Diffraction

Electron diffraction patterns obtained from the ferritins isolated from the Australian β -thalassemic spleen and Thai β -thalassemic/Hb E spleen both indicated the

presence of the mineral ferrihydrite. The diffraction patterns were consistent with those measured by Haggis [86] and Harrison *et al.* [89] for ferritin from an unspecified source. The pattern from the Thai β -thalassemic/Hb E spleen ferritin indicated that the degree of crystallinity of the mineral present may be somewhat lower since a larger number of the reflections tended to be missing from the patterns measured.

The hemosiderin isolated from the Australian β -thalassemic spleen gave electron diffraction patterns that indicated the presence of both ferrihydrite and a mineral with a higher degree of crystallinity. The d-spacing of 2.73 Å is close to those observed in hemosiderins isolated from other organs taken from multiply transfused thalassemic patients [120] which were identified as being related to the structure of goethite (α -FeOOH). Although a d-spacing of 3.55 Å has not been previously reported for tissue iron oxide deposits, it may possibly be related to the goethite structure, since goethite shows a d-spacing of 3.38 Å and the errors on measurement of d-spacings are somewhat larger for the larger d-spacings. Thus the electron diffraction patterns for this hemosiderin sample most likely indicate the presence of the goethite like form of hemosiderin.

Table 5.2 shows a summary of the fraction of the Mössbauer spectrum of each of these samples (along with other relevant samples) at 78 K, that is in the form of a sextet together with the phases identified by electron diffraction. These data confirm the assertion that the presence of a significant sextet signal in Mössbauer spectra of mammalian tissues or tissue extracts at 78 K is indicative of the presence of the goethite like form of iron oxide in the specimen. Although the dietary iron loaded rat liver showed a significant sextet signal at 78 K, the signal was small suggesting that the quantity of goethite like iron oxide in the liver section may be below the detection limit for electron diffraction techniques.

Iron Oxyhydroxide Particle Size

Evidence supporting the view that hemosiderin may be derived from ferritin is the smaller size of the hemosiderin iron oxyhydroxide core [93,106,118,151]. TEM results (Figures 5.11 and 5.13) show that the average core size of crude hemosiderin is smaller than that of ferritin, supporting these previous results. Nevertheless, hemosiderin, fractionated from crude hemosiderin, showed a high variation of core sizes compared to those of the isolated ferritins. This observation indicates the heterogenous nature of hemosiderin, which again suggests that it may originate from more than one mechanism.

From the Mössbauer study at 78 K, the crude spleen hemosiderins, isolated from chelated and transfused β -thalassemic patients, are composed of at least two forms of iron oxide including a high proportion of the goethite like form of hemosiderin (Figure 5.6b). The crude hemosiderin with a wide range of particle size (Figure 5.12) showed the highest proportion of the goethite like form of hemosiderin. Fortunately, the discrete separation of the three populations of particles enabled further examination. In general, these particles exhibit quite different appearance

Table 5.2 Comparison of electron diffraction data with Mössbauer spectral data for specimens at 78 K.

Specimen	Phases detected by electron diffraction	Fraction of sextet in Mössbauer spectrum at 78 K
(a) Aus thal ferritin	Fh	0.08 ± 0.07
(b) Thai thal ferritin	Fh	0.04 ± 0.04
(c) Ferritin crystal	Fh	0.03 ± 0.01
(d) Aus thal hemosiderin	Gt + Fh	0.60 ± 0.08
(e) Dugong liver	Gt + Fh	0.44 ± 0.03
(f) Rat liver	Fh	0.08 ± 0.02
(i) Thal Ferritin	Fh	
(j) Thal liver hemosiderin	Gt + Fh	~ 0.50
(k) Thal spleen hemosiderin	Gt + Fh	~ 0.50
(l) PH liver hemosiderin	Fh	no sextet
(m) Horse hemosiderin	Fh	no sextet

Fh = ferrihydrite; Gt = goethite like iron oxide

- (a) Australian β -thalassemic spleen ferritin.
- (b) Thai β -thalassemic/Hb E spleen ferritin.
- (c) Horse spleen ferritin crystal fixed and embedded in resin.
- (d) Australian β -thalassemic spleen haemosiderin.
- (e) Dugong liver tissue section fixed and embedded in resin.
- (f) Dietary iron loaded rat liver (15 months old) section fixed and embedded in resin.
- (i) Thalassemic (transfused) ferritin from liver and spleen [120].
- (j) Thalassemic (transfused) liver hemosiderin [120].
- (k) Thalassemic (transfused) spleen hemosiderin [120].
- (l) Primary hemochromatosis liver hemosiderin [120].
- (m) Horse spleen hemosiderin [123].

and size. These differences may be related to the presence of different forms of iron oxyhydroxide in this sample, although the finding was only specific to this particular sample. However, smaller size particles in the range of 1–2 nm were observed in another sample of a β -thalassemic spleen hemosiderin (Figure 5.11b).

The finding of the variation of the core size in crude hemosiderin samples may have resulted from the use of crude hemosiderin. Small electron dense particles (2–4 nm) in addition to the typical ferritin cores have been occasionally seen in the cytosol of iron laden cells [41]. Besides, *in situ* measurements of the hemosiderin core in iron overloaded liver cells indicate that the particle size ranges from approximately 0.8 nm to 7 nm in diameter [63]. It has been shown that the amorphous iron oxide fraction tends to be lost in the process of further purification using high density (KI) centrifugation [126]. The correlation between the core size and the type of iron oxides present in hemosiderin deserves further investigation.

Infrared Spectroscopy

Many organic components of hemosiderins, such as lipid [60,113,146] in the form of free cholesterol, phospholipid, free fatty acid, long chain fatty acid, triacyl glycerol and phospholipid have been previously reported [60]. The present IR investigation of isolated fractions demonstrates that organic components can be identified.

Proteins exhibit a common pattern of absorption bands, including those due to hydrogen bonded NH groups at 3300 cm^{-1} and 3080 cm^{-1} and the amide I and II bands in the $1600\text{--}1500\text{ cm}^{-1}$ region [149,152]. The amide (I and II) absorptions which appear in all polypeptides and proteins, were also observed in these three isolated iron storage samples (Figures 5.9 and 5.14). The majority of these materials absorb in the normal 1650 cm^{-1} and 1550 cm^{-1} regions. The strong amide I band (1700 cm^{-1} to 1620 cm^{-1}) is due almost entirely to the C=O stretching vibration of the peptide linkage. The amide II band (1600 cm^{-1} to 1500 cm^{-1}) is due to an out of phase combination of N–H in plane bending and C–N stretching vibrations of peptide groups. Minor differences that occur amongst the absorption regions of amide I and II bands to some extent may reflect changes in the hydrogen bonding pattern [149,152]. The ratio of the intensity of the peaks at 1650 cm^{-1} and 1540 cm^{-1} differs among these isolated iron oxide samples. This variation may be due to H_2O binding at 1640 cm^{-1} , which occurs under the amide peak.

In the region of 3000 cm^{-1} , IR absorption bands arise from C–H stretching modes of organic materials contained in ferritin and haemosiderin. Partly contributed to by H_2O , a group of absorption bands in the 3400 cm^{-1} region is due to stretching vibrations of the OH bond. Two strong bands occurred at 2926 cm^{-1} and 2853 cm^{-1} . These bands correspond to the in phase and out of phase vibrations of the hydrogen atoms in the CH_2 groups present in hydrocarbons [149]. The characteristic C–H deformation frequencies were identified in the regions of 1453 cm^{-1} and 1394 cm^{-1} . The C–O stretching vibration was found at the absorption frequencies of 1240 cm^{-1} and 1060 cm^{-1} . This region may include absorptions distributed by long chain fatty acids which exhibit a regular series of evenly spaced absorptions in the region of $1350\text{--}1180\text{ cm}^{-1}$ when examined in the solid state [152]. As specified earlier, some of these peaks may be due to phosphorus compounds which show absorption peaks in this range.

Metal oxides have absorption bands in the extended mid and far infrared regions (less than 800 cm^{-1}), owing to metal oxygen stretching or lattice vibrations [153]. A strong band assigned to inorganic phosphate (PO_4^{3-}) is found in the region $1050\text{--}1000\text{ cm}^{-1}$, together with a second absorption in the $900\text{--}980\text{ cm}^{-1}$ region in some cases where the symmetry is disturbed [149,154]. The distinct 960 cm^{-1} peak showed relatively higher absorption in the β -thalassemic spleen hemosiderin compared to the other hemosiderins (Figure 5.14f). This observation corresponds well to the finding of the high P:Fe ratio as measured by ICP. Although the iron concentration of this sample is low, this distinctive peak assigned to phosphate as well as the lower energy peaks (890 cm^{-1} , 795 cm^{-1} and 640 cm^{-1}) assigned to the goethite present, indicate that the inorganic components may be less associated with the organic components. This 960 cm^{-1} peak also appeared in the spectrum of the liver hemosiderin treated with DMSO (Figure 5.9e), to remove some organic components. This observation suggests that the phosphate is bound to the hemosiderin cores.

The discovery of the goethite peaks in the isolated spleen hemosiderin (Figure 5.14f) is consistent with the Mössbauer spectroscopic results. The well

defined peaks corresponding to Fe–O lattice vibrations in goethite were seen at low frequency (647 cm^{-1} and 464 cm^{-1}), along with the distinctive O–H bending doublet at 794 cm^{-1} and 889 cm^{-1} [155]. The faint peak due to O–H stretching in goethite around $3140\text{--}3100\text{ cm}^{-1}$ was also observed in the synthetic goethite (Figure 5.14b) near the O–H stretching peak of H_2O in 3407 cm^{-1} leading to a broader peak than one found in synthetic ferrihydrite, which showed only the H_2O absorption.

The removal of organic components by using an organic solvent (DMSO) and UV exposure, in combination with ultrasonic agitation, was not complete. When a crude liver hemosiderin was treated with UV light, no discernible changes in absorption peaks were observed. After further treatment with DMSO, the minor absorption peak at 960 cm^{-1} owing to phosphate absorption was noticeable.

These spectral results indicate that only a small (if any) organic fraction has been extracted from the crude hemosiderin. As UV treatment in the presence of air supports free radical reaction, it would appear that the organic components are exceedingly stable. This stability suggests that the organic components are part of the composition of hemosiderin isolated in this manner. Details of the relationship of these organics and the inorganic iron oxides remain unclear.

Iron and Phosphorus Content

Crude human hemosiderin samples used in this study showed a wide variation of the concentration of iron and of the P:Fe ratio (0.2–18). These values are larger than those reported for two separate samples of human β -thalassemic hemosiderin namely 1:6.0 and 1:14.9 [112]. The greater fractions of phosphorus in our samples are most probably due to the generally larger organic component in crude hemosiderin compared with KI density centrifugation isolated hemosiderin. The concentration of phosphorus was in the range of 0.7–2.3 % with no correlation with the concentration of iron (0.1–9.0 %). Most of the crude hemosiderin preparations from the Thai β -thalassemic/Hb E liver and spleen have P:Fe ratios lower than 1. The crude hemosiderin from the Australian β -thalassemic patients have P:Fe ratios of more than 1. Interestingly, in the case of crude dugong hemosiderin, the P:Fe ratio is low (0.08). In this study no phosphate buffered saline (PBS) was used in the isolation of ferritin and hemosiderin. The changes in the P:Fe ratios of the crude hemosiderin may correspond to the source of hemosiderin present and/or the amount of organic phosphate present in the sample.

The P:Fe ratios for the ferritin isolated from thalassemic livers and spleens of this study fall in the range of previous reports (P:Fe = 0.1–0.3) [125]. It is interesting to note that the ferritin isolated from the Australian β -thalassemic spleen has a very low ratio of 0.07, although its iron concentration is close to that of the Thai β -thalassemic/Hb E spleen ferritin. In addition, this spleen ferritin shows the largest iron oxyhydroxide core sizes when compared to those of Thai β -thalassemic/Hb E spleen (and liver ferritin) (Figure 5.13). Diffraction data (Table 5.1) suggest that the crystallinity of this ferritin may be higher than that of the Thai β -thalassemic/Hb

E spleen ferritin (electron diffraction of Thai β -thalassemic/Hb E liver ferritin not available).

In this study it was found that the higher the iron concentration in the tissues, the higher was the iron concentration in the crude hemosiderin fraction (Figure 5.10). This observation may explain why previous studies showed such a wide range of iron concentrations in hemosiderins. Previous studies of iron concentrations in hemosiderins isolated from hemosiderotic livers (KI method) reported values in the range 7.6–36 % [146]. With high density centrifugation using LiCl [142], hemosiderin with 9 % iron was obtained. The lower iron content of isolated granules may indicate either some contamination or a higher amount of other cell constituents. Further purification of hemosiderin beyond the crude hemosiderin stage (e.g. the KI centrifugation) results in higher iron fractions in the hemosiderin but the iron concentrations are still correlated with the original tissue iron concentrations (Figure 5.10). This phenomenon has been previously observed [156]. In addition, there appears to be a correlation between the fraction of iron in the goethite like form of iron oxide in hemosiderin and the fraction of iron in the goethite like form of iron oxide in the tissue from which the hemosiderin was isolated (Figure 5.10b). There is a datum point from a previous study [132] which has been included in Figure 5.10b and which falls within the observed correlation. This previous Mössbauer spectral study at 78 K of the hemosiderin purified by KI centrifugation method showed a Mössbauer spectrum with a sextet area of 28 % (i.e. $F_s = 0.28$). This hemosiderin had been isolated from a spleen whose Mössbauer spectrum had 25 % sextet area (i.e. $F_s = 0.25$). This correlation is to be expected if the hemosiderin isolated from the tissues is representative of the predominant form of iron in the tissues.

5.3 ACCESSIBILITY OF HEMOSIDERIN IRON TO AN IRON CHELATOR

Knowledge of the mechanisms of iron release from the iron storage compounds, ferritin and hemosiderin, is of importance in understanding both the pathology of tissue damage associated with iron overload and therapeutic treatment in chelation therapy. The release of iron from ferritin has been studied extensively [156–164]. Iron release from hemosiderin isolated from primary hemochromatotic and thalassemic liver and spleen also has been studied to some extent [93,124,156,160,165–167]. However, the different types of iron oxides existing in isolated hemosiderin have not previously been studied in relation to the reaction of these various forms of hemosiderin with iron chelators.

Crude hemosiderin isolated from various thalassemic tissues, as described in the previous section, was used to study the effect of chemical speciation of hemosiderin iron on iron release. Since there is no procedure available for isolating the different forms of tissue iron oxides, representative tissues with various fractions of the goethite like form of hemosiderin were used as sources of the hemosiderin isolates. For comparison, samples of the synthetic iron oxides ferrihydrite and goethite were

used as controls in dissolution studies using the iron chelator, desferrioxamine (DFO). Under biological conditions, the goethite like form of hemosiderin is present as a mixture together with the ferrihydrite like form of hemosiderin. A control consisting of a mixture of synthetic ferrihydrite and goethite was also studied so as to evaluate the contribution of the organic components in the tissue to the experimental results. Crude dugong liver hemosiderin was used as the comparison material for the goethite like form of hemosiderin isolated from iron overloaded liver tissue. It has been shown that this hemosiderin is composed of a very high percentage of iron (low contamination by organic component) and a large fraction of goethite like hemosiderin (see earlier). This study used crude hemosiderin so as to ensure that all the water insoluble iron components were included [126,147].

Mössbauer spectroscopy has confirmed the presence of ferrihydrite and goethite like iron in livers and spleens from thalassemic patients [135]. The different crystal structures of the ferrihydrite and goethite like forms of hemosiderin, and their differing degrees of crystallinity may affect their rates of dissolution and, hence, the rate of iron release from tissue. Therefore, the rate of dissolution of iron from crude hemosiderin was determined *in vitro* using dialysis. Such *in vitro* studies on the removal of iron from hemosiderin are not only of interest for structural reasons, but any insights gained into the mobility of hemosiderin iron are relevant to an understanding of iron metabolism and the relative toxicity of this iron. From a clinical perspective, such studies on the mobilization of hemosiderin iron may lead to refinements in the treatment of iron overload.

5.3.1 MATERIALS AND METHODS

Samples

The hemosiderins from two β -thalassemic/Hb E livers, two β -thalassemic/Hb E spleens and two β -thalassemic spleen tissues, and isolated ferritins and synthetic iron oxides, studied in the previous section, were used in these experiments. A mixture (1:1 molar ratio of iron) of synthetic iron oxides (ferrihydrite and goethite) was also studied.

Dissolution of Iron Oxide Using DFO

Desferrioxamine, in the form of its methanesulfonate salt (Desferral[®], Ciba-Geigy Ltd., Basel, Switzerland) was the iron chelator used throughout this study. Tris/HCl buffer (pH 7.4), prepared from tris-hydroxymethylaminomethane (ultrapure, Boehringer[®]) was used as the buffer in the system. Tris/HCl buffer was sterilized before use by passing through a 0.25 mm Millipore[®] filter.

Iron release to DFO was determined by the dialysis method [156]. Dialysis tubing with a 12 000–14 000 molecular weight cut-off (Selby Scientific Ltd.) was treated before use by boiling with 5 % ethanol for 15 min, then with 1 mM EDTA for a further 30 min so as to remove metal ions. Finally, the tubing was boiled

twice more with Millipore® water for 30 min each. The tubing was finally washed with Millipore® water to remove all contaminants adhering to the outside and was then kept in a clean container of Millipore® water. All glassware in this experiment was acid washed with 30 % HCl to remove any contaminating iron and other metals.

The molar ratio of iron to DFO was fixed at 1:20 [156]. Synthetic iron oxides and isolated ferritin and ‘solubilized’ crude hemosiderin samples were appropriately diluted to produce suspensions of 1 mM iron in 50 mM Tris/HCl buffer at pH 7.4. Each suspension was mixed well before transferring into the treated dialysis tubing. Each suspension was prepared in triplicate. A 1 mM solution of DFO was prepared using sterilized 50 mM Tris/HCl buffer. In these experiments 5 ml of the 1 mM iron suspension were dialyzed against 100 ml of the 1 mM DFO solution. These volumes were five times larger than those used by O’Connell *et al.* [156] in order to enable the collection of larger samples, thereby minimizing sampling errors. The experiment was performed in sterile containers with constant agitation at a speed of 150 cycles per min on a horizontal platform shaker (Certomat®) at 37 °C, over a period of 25 days.

The dissolution reaction was followed by measuring the amount of iron released at regular time intervals of 24 hours. Samples of dialysate (1 mL) were removed and the release of iron to DFO in the dialysate assessed by measuring the concentration of the iron–DFO complex (ferrioxamine). The absorbance of the iron–desferral complex was measured at 430 nm using a Hewlett Packard UV-Vis spectrophotometer. The spectrophotometer was fitted with a waterbath, set at 25 °C. The reliability of the spectrophotometer was calibrated with a holmium oxide (Ho₂O₃) glass verification sample before measurement. The percentage of iron released was calculated from the absorption data and atomic absorption spectrometry, after correction for changes in the volume of the dialysate. At the conclusion of dissolution experiments, the retentate remaining from one sample of hemosiderin isolated from an Australian β -thalassemic spleen, was removed from the reaction, dialyzed with Millipore® water and freeze dried. This sample was examined by Mössbauer spectroscopy to determine which fraction of iron oxide, the ferrihydrite like or goethite like form, was dissolved by DFO.

5.3.2 RESULTS

The percentage of iron released from hemosiderin isolated from various tissues after 25 days varied from approximately 22 % to 79 % (see Figure 5.15). Crude hemosiderins, isolated from the β -thalassemic/Hb E spleens, tended to release more iron than those isolated from β -thalassemic/HbE livers and β -thalassemic spleens (Figure 5.15). Hemosiderin isolated from an Australian β -thalassemic spleen released the lowest percentage of iron (22 %).

The extent of iron release from the isolated hemosiderins is inversely related to the percentage of sextet area obtained from their respective Mössbauer spectra (Figure 5.16). The proportion of goethite like to ferrihydrite iron in the hemosiderin

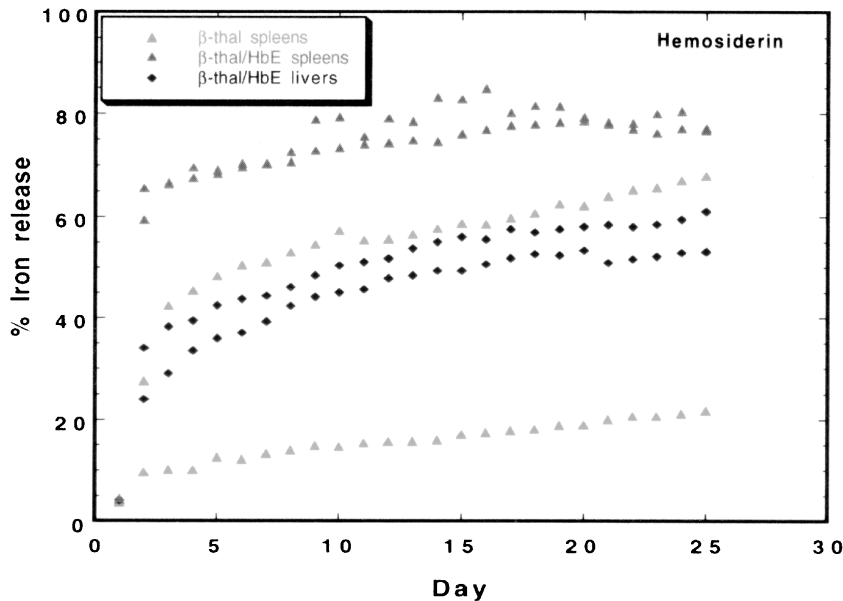


Figure 5.15 Iron released (%) from crude hemosiderin isolated from spleens of β -thalassemic and β -thalassemic/Hb E patients and from livers of β -thalassemic/Hb E patients.

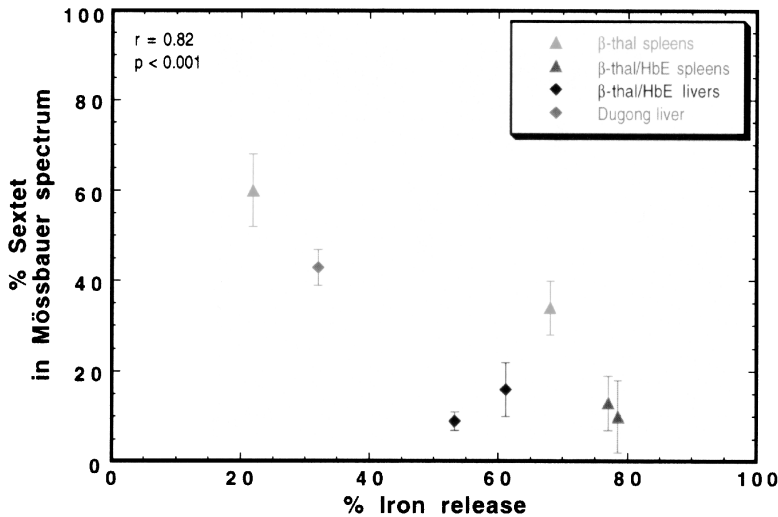


Figure 5.16 Correlation between the percentage of hemosiderin iron released and the relative fraction of iron in the form of the goethite like iron oxide, as measured from the Mössbauer spectra.

samples is related to the ratio of the area of the Mössbauer sextet to the area of the Mössbauer doublet. Figure 5.16 shows the percentage of iron released by the hemosiderin samples vs the fraction of sextet in the Mössbauer signal at 78 K (i.e. a measure of goethite like iron fraction within the sample). The percentage of iron released from these crude hemosiderins was closely related to the percentage of sextet area from the Mössbauer spectra, with a correlation coefficient of $-0.82 (p < 0.001)$. Therefore, it appears that hemosiderin samples containing a high percentage of the goethite like form release less iron than do samples containing a low percentage of the goethite like form. The crude hemosiderin isolated from an Australian β -thalassemic spleen showed a higher percentage of sextet area and, in turn, released less iron than hemosiderin isolated from β -thalassemic/Hb E spleens and livers. This observation, together with the data on the chemical speciation of iron in the spleens of transfused and chelated Australian β -thalassemia patients and nontransfused nonchelated Thai β -thalassemia/Haemoglobin E patients [135], suggests that regular blood transfusion and iron chelation therapy may result in a relatively higher percentage of iron being stored in the form of goethite. For comparison, data are included in Figure 5.16 for dugong liver hemosiderin, containing a high proportion of the goethite like form.

The Mössbauer data from an Australian β -thalassemic spleen hemosiderin show a high percentage of the goethite like form of iron oxide. The freeze dried hemosiderin, after 25 days of dialysis with DFO, was studied by Mössbauer spectroscopy in order to determine which form of iron oxide may have been released. Figure 5.17 shows that the relative area of the sextet (F_s) from the dialysate residue is 0.61 ± 0.09 in comparison to that of the original sample which was 0.60 ± 0.08 . As a result, there was no detectable preferential mobilization of iron from either the ferrihydrite or goethite like forms of iron oxide. However, since only 22 % of the iron was mobilized, the uncertainties on the values of F_s in the measurements do not rule out the possibility of preferential mobilization. Unfortunately, not enough sample was available to enable more detailed Mössbauer measurements.

The difference in the ease of dissolution of the goethite like and ferrihydrite like forms of hemosiderin was consistent with results obtained from each of the synthetic iron oxides and their mixture (Figure 5.18).

The data indicate clearly that ferrihydrite releases significantly more iron than does goethite. Over 25 days, ferrihydrite released 72.3 % of its iron content, while goethite released 5.7 % of its iron, approximately 13 times less than ferrihydrite. Interestingly, a mixture of the synthetic iron oxides, in a 1:1 molar ratio of iron, released only 18 % of its iron content. The TEM images of the iron oxide mixture, before and after DFO dissolution, are shown in Figure 5.19. The goethite has a very similar appearance both before and after treatment with DFO.

Figure 5.20 shows the iron release data obtained from ferritin isolated from β -thalassemic/Hb E livers, β -thalassemic/Hb E spleens and the β -thalassemic spleens. In general, the percentage of iron released ranged from 38 % to 55 %.

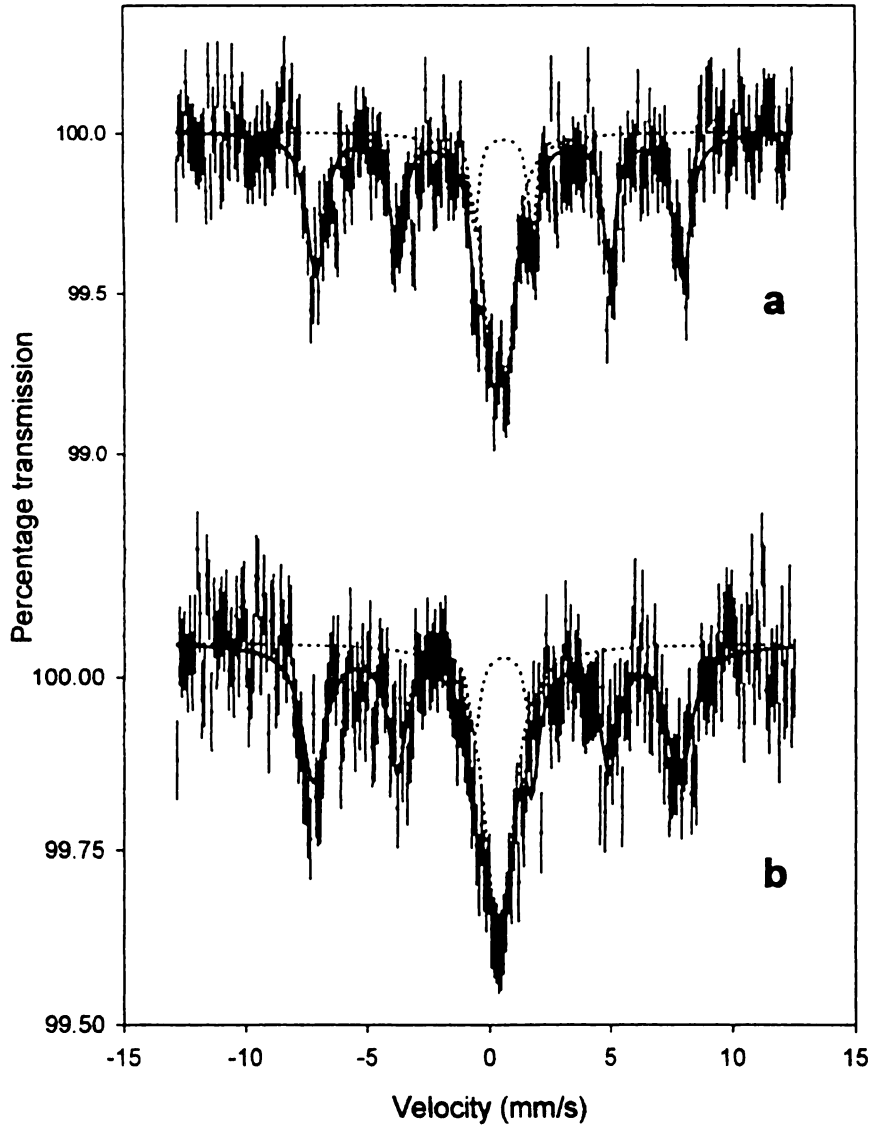


Figure 5.17 Mössbauer spectra of a crude hemosiderin isolated from a β -thalassemic spleen (a) before and (b) after reaction with DFO for 25 days (sample temperature 78 K).

It can be seen that β -thalassemic/Hb E liver ferritin released more iron than both β -thalassemic/Hb E spleen and β -thalassemic spleen ferritins. The Australian β -thalassemic spleen ferritin released the least amount of iron. Comparison of the ferritin data with that of synthetic ferrihydrite shows that ferritin releases less

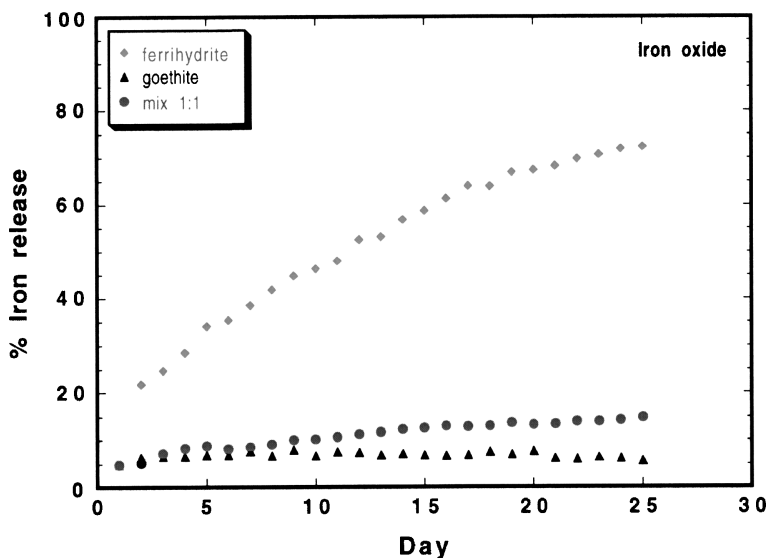


Figure 5.18 Iron release from synthetic iron oxides by treatment with DFO.

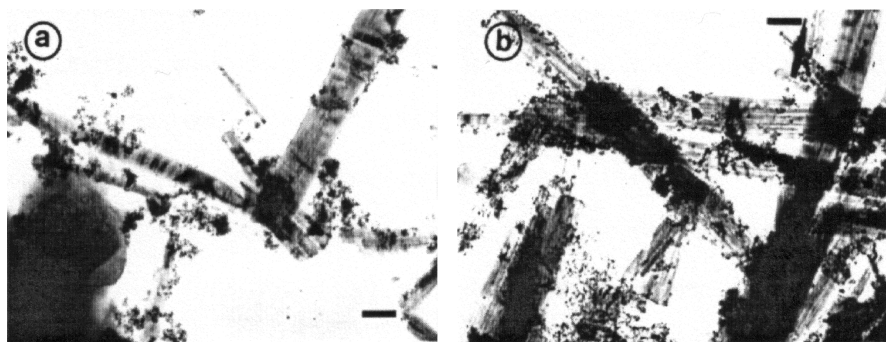


Figure 5.19 TEM images of the mixture of ferrihydrite and goethite (a) before and (b) after 25 days of the reaction with DFO (scale bar is 100 nm).

iron than does synthetic ferrihydrite (Figure 5.20). Since ferritin is, in essence, ferrihydrite contained within a protein shell, the observed difference between the amounts of iron released may be attributable to the presence of the protein component of ferritin. This observation is consistent with recent studies on iron release from ferritin while controlling the conformation of the ferritin shell pores [71].

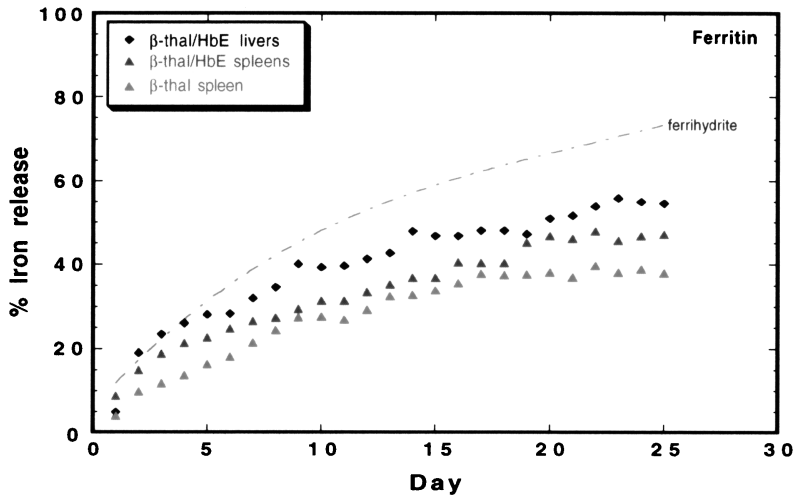


Figure 5.20 Iron release from ferritin isolated from β -thalassemic/Hb E liver and spleens, ferritin isolated from β -thalassemic spleen and synthetic ferrihydrite.

Figure 5.21 illustrates the TEM images of β -thalassemic/Hb E liver ferritin along with particle size distribution data both before and after dissolution. The particle size distribution data show that the average particle size of the ferritin decreased from 6.10 nm to 4.93 nm, indicating that the iron was removed from the protein shell. By assuming that the density of iron within the cores is uniform and that the cores have a spherical shape, the measured decrease in diameter suggests that approximately 50 % of the iron remains after the dissolution process, which is consistent with the elemental analysis data.

In comparison with the hemosiderin, the ferritin generally released more iron than the hemosiderin that contained a high fraction of goethite like iron (Figure 5.22). However, many of the other hemosiderins released more iron than the ferritin (Figure 5.15).

5.3.3 DISCUSSION

The amount of iron released from the hemosiderins varied according to the tissue sample, that is, the organ from which the tissue originated, and the clinical treatment received by the patient. The ability of hemosiderins, isolated from tissues subject to various conditions of iron overload, to release iron to iron chelators has been investigated by various research groups [93,124,156,160,165,166].

The present study has indicated that the differences in the extent of iron mobilization in hemosiderin may be due, at least in part, to differences in the chemical form of iron oxide comprising the mineral core. The Mössbauer

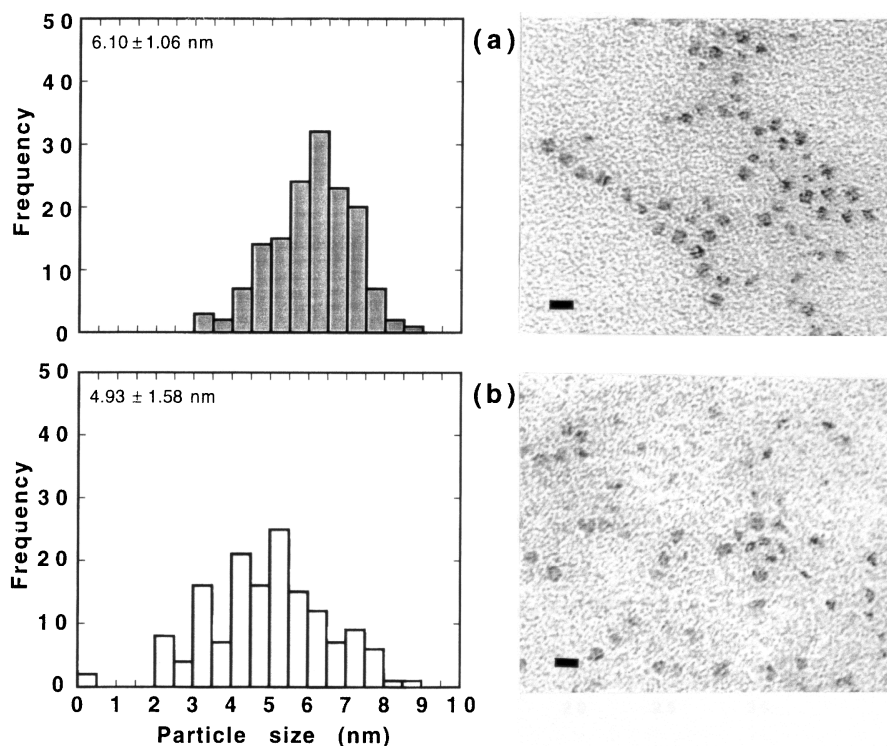


Figure 5.21 TEM images and particle size distribution of ferritins isolated from β -thalassemic/Hb E liver (a) before and (b) after 25 days of reaction with DFO.

spectroscopic results and the iron release data from the different hemosiderins indicate that hemosiderins with a high percentage of the goethite like form of iron oxide release less iron than do those with a relatively higher percentage of the ferrihydrite form (Figure 5.16). The ferrihydrite form of iron oxide tends to allow iron to be mobilized more readily than does the goethite like form.

The relationship between the availability of iron and the crystal structure of the iron oxide is consistent with the iron release experiments using synthetic iron oxides (Figure 5.18). These experiments also show that there are considerable differences in the degree of iron release, depending upon the form of the iron oxide (Figure 5.18). In the reaction with desferrioxamine as the iron chelator, the ferrihydrite form releases more iron than does the goethite form of iron oxide. This observation can be explained in terms of the structure of these two forms of iron oxide. Ferrihydrite forms as very small particles of low crystallinity and the large surface area makes this iron oxide phase highly reactive in comparison to goethite, which forms larger crystals (Figure 5.8). In addition, the solubility product of ferrihydrite is approximately 10^{-36} to 10^{-39} compared with 10^{-42} to 10^{-44} for goethite [5]. The

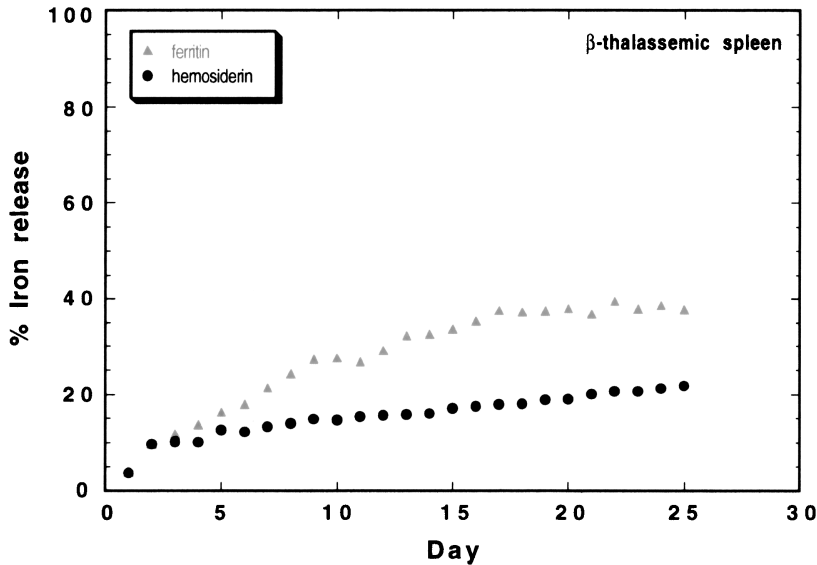


Figure 5.22 Iron release from ferritin and hemosiderin isolated from a β -thalassemic spleen.

synthetic iron oxides used in this study may not, however, closely resemble those occurring in the tissues. In the case of hemosiderin, the iron oxide is generally mixed with organic matter such as fatty acids, carbohydrate and proteins [112,145].

Comparisons between the iron mobilization from ferritin and hemosiderin have been studied previously [124,156,160,165,166]. Kontoghiorghe *et al.* [160] showed that hemosiderin isolated from an iron overloaded thalassemic spleen releases more iron than does ferritin obtained from the same source. This finding suggested that the iron pool in hemosiderin is more labile than that in ferritin. However, later observations by Andrews *et al.* [93], O'Connell *et al.* [156] and Ward *et al.* [124] have shown that hemosiderin isolated from human spleens removed from patients suffering from thalassemia major, and who had received multiple blood transfusions, released iron to DFO less than did spleen ferritin from the same source. In their publication, O'Connell *et al.* [156] suggest that the isolated hemosiderin used by Kontoghiorghe *et al.* [160] had a low iron content. The study by Ward *et al.* [124], which showed that hemosiderin isolated from thalassemic spleens and livers released less iron than thalassemic ferritin, also showed that hemosiderin, isolated from primary hemochromatosis livers, released more iron than primary hemochromatosis ferritin. In addition, Andrews *et al.* [93] also reported that, for rat liver, the percentage iron released from hemosiderin was equal to or greater than that from ferritin. These iron oxide deposits were isolated from rat livers that had been loaded by a single intraperitoneal injection of iron-dextran.

Most of the hemosiderins isolated from thalassemic tissues in this study agree with the results of Kontoghiorghe *et al.* [160], in which hemosiderin iron is more

available than ferritin iron. The findings to the contrary, reported in previous studies [93,156,166] may also be explained by the results of this study. One of the hemosiderin samples, isolated from the Australian β -thalassemic spleen, shows a lesser degree of iron dissolution in comparison to ferritin. This hemosiderin sample has a high variation in the sizes of iron cores and a very high percentage of the goethite like form of iron (Figures 5.6 and 5.12). The availability of iron in hemosiderin is also dependent upon the percentage of goethite in the samples present. It is possible that the samples used in previous reports [93,156,166] contained a higher percentage of the goethite like form of hemosiderin than the spleen sample used by Kontoghiorghes *et al.* [160].

Differences between hemosiderin and ferritin in the phosphate content of their iron oxyhydroxide cores, in core size, and in both the amount and type of organic components may also account for the differences in the degree of iron dissolution. The effect of iron oxyhydroxide crystallinity on iron release has been evaluated using the mineral core of ferritin. The ferritin core is composed of the one type of iron oxyhydroxide, ferrihydrite, which possesses a semirandom structure [59]. The crystal structure of ferritins range from noncrystalline to comparatively highly crystalline, depending on their origin and mode of formation [92]. The crystallinity of the iron oxyhydroxide core is also partly related to the P:Fe ratio [92] with a very high phosphate content of the ferritin core being associated with decreasing core crystallinity. The variation in crystallinity amongst human ferritin has been found to be limited [150]. The relationship between the amounts of iron mobilized from these ferritin samples by DFO in this study is as follows:

β -thal/Hb E liver ferritin > β -thal/Hb E spleen ferritin > β -thal spleen ferritin

The relationship between the P:Fe ratios of this set of samples follows the same trend, that is:

β -thal/Hb E liver ferritin > β -thal/Hb E spleen ferritin > β -thal spleen ferritin

Therefore, in this study, the availability of iron in ferritin is correlated to the P:Fe ratio and hence, to the crystallinity of the ferritin core. Highly crystalline ferrihydrite releases less iron than does ferrihydrite with low crystallinity. Hence, the β -thalassemic spleen ferritin shows the lowest amount of iron release, has the lowest P:Fe ratios and the highest crystallinity.

The presence of organic components may affect the availability of the iron oxide deposit to the chelator. Ferrihydrite without a protein shell shows a greater availability of iron for dissolution than it does in the ferrihydrite core of ferritin (Figure 5.20). This observation is consistent with previous findings [89]. Ferrihydrite inside the protein shell of ferritin is less accessible to the iron chelator. In addition, Kontoghiorghes *et al.* [160] showed that iron(III) precipitates are able to release iron to iron chelators more readily than do ferritin and hemosiderin.

Hemosiderin isolated from different iron loading syndromes, such as primary hemochromatosis (PHC) and secondary hemochromatosis (SHC) (thalassemia), has

been reported to exhibit differences in both its degree of iron release and its peptide composition [124]. It has been commented that the relative integrity of the polypeptide matrix in hemosiderin in PHC livers compared to that of SHC liver hemosiderin may be implicated in the higher rate of release of iron(III) from the PHC liver. Thus, retention of the matrix may facilitate the reductive release of iron from the core as well as maintaining a greater degree of solubility of the core [124].

Ferritin and hemosiderin are reportedly able to stimulate hydroxyl radical formation [156,165,166,168,169], lipid peroxidation [170] and protein modification [169], by mechanisms involving iron release. The greater the availability of iron, the more effective the promotion of free radicals [171]. Some hemosiderin preparations have been shown [156,166] to be less active than ferritin because the mobilization of iron from these hemosiderins was more difficult than for the ferritin studies. Hemosiderin, however, can provide iron to participate in the generation of $\text{OH}^\bullet$ radicals. Hemosiderin is less effective than ferritin in promoting hydroxyl radical formation or stimulating lipid peroxidation in the presence of ascorbate [165]. These findings suggest that changes in the relative amounts of iron stored as ferritin to iron stored as hemosiderin is biologically advantageous [165]. However, none of these previous studies have considered whether the various forms of iron oxide present in hemosiderin could be related to their relative iron toxicity. The availability of iron in hemosiderin most likely depends upon the type of iron core present, which needs to be identified and compared to the core in ferritin, in order to fully evaluate these previously reported data.

Relating some of the individual case histories with the results presented in this study serves to clarify the relationships among iron loading, forms of iron present, toxicity and clinical history. Thus, a high percentage of iron released in both cases of β -thalassemic/Hb E spleen hemosiderins considered in this study can be related to their high proportion of the ferrihydrite like form of hemosiderin present. These hemosiderins were isolated after autopsy from spleens of Thai patients (aged 30 and 36 years) who had received a total of 35 units of red blood cells without chelation therapy. The iron concentration of these spleens was 6.3 mg g^{-1} and 10.0 mg g^{-1} dry weight, respectively. The spleen with the higher iron concentration had been removed from the older patient. Isolated hemosiderin samples from these spleens, show both a high percentage of iron release and a high percentage content of the ferrihydrite like form of hemosiderin.

Samples were available of ferritin and hemosiderin isolated from an Australian β -thalassemic spleen from a patient, aged 11 years, who underwent regular blood transfusion and DFO treatment. The concentration of iron in the spleen of this particular patient was approximately 4.0 mg g^{-1} dry weight. The spleen tissue and its hemosiderin had a high percentage content of the goethite like form of hemosiderin in conjunction with less iron release from its hemosiderin. This hemosiderin showed less iron release compared with other isolated hemosiderins and the ferritin isolated from the same spleen. A higher degree of iron release was found in the other Australian β -thalassemic spleen, removed from a patient aged 8 years. This spleen has an iron concentration of 8.0 mg g^{-1} dry weight with $F_s = 0.32$, about half the

value found in the other Australian β -thalassemic spleen. The variation found in these Australian β -thalassemic spleens may be due to the age of the patients, duration of the treatment and also individual variability. The lower iron concentration in the spleen tissue found in the older patient may reflect a history in which the patient has undergone longer treatment with iron chelation therapy and regular blood transfusion. These findings suggest that the treatment with DFO may not only remove extra iron received from regular blood transfusion, but may also shift the relative amount of the more available iron oxyhydroxide (ferrihydrite) to a more stable form (goethite). No sign of hemosiderosis damage was observed in the Australian β -thalassemic spleen (Figure 5.7). This raises the possibility that the goethite like hemosiderin is of biological advantage to the cell. The transformation of iron in hemosiderin into the more stable form of goethite like hemosiderin may be beneficial in avoiding tissue damage.

5.4 CONCLUDING REMARKS

The data reviewed and reported in this chapter have established that there is significantly different chemical speciation of iron in tissues between identifiable different groups of patients [135]. Iron overloaded patients who had undergone regular blood transfusion and chelation therapy tended to have a high fraction of nonheme storage iron as the goethite like form of hemosiderin. In contrast, the iron overloaded patients who had minimal blood transfusion without chelation therapy tended to have a higher fraction of the ferrihydrite like form of hemosiderin.

Hemosiderin is a heterogenous iron storage component with variations in the organic components, particle size, iron concentration and also the chemical form of iron oxyhydroxide present. The different forms of iron oxyhydroxide in hemosiderin have shown different availability to the iron chelator, desferrioxamine. Future work will need to evaluate the availability of the iron from these hemosiderins to new chelators such as deferiprone.

The different chemical speciation of iron in tissues between different identifiable groups of patients has implications for the treatment and management of iron overload diseases. The reactivities of the different hemosiderin iron forms are different. Although the goethite like form of hemosiderin is likely to be more soluble than goethite itself, hemosiderin from transfusional iron loaded patients has been shown to release iron less readily than the ferrihydrite cores of ferritin when exposed to the iron chelator. Thus, the ferrihydrite form of hemosiderin may be more toxic to cells than the goethite like form on an atom for atom basis because of its higher solubility. On the other hand, it should be easier to chelate and remove from the body than the goethite like form. This hypothesis may partly explain why the Australian patients reviewed in this chapter had higher fractions of their nonheme spleen iron in the goethite like form. More of the ferrihydrite form may have been removed by the chelation therapy, the remaining deposits thus being enriched in the goethite like form.

The conclusions drawn here have implications for future directions in the management of iron overload diseases. For example, in the research and development of new iron chelators, it should be taken into account that the different forms of iron oxyhydroxide deposit may have different affinities for a given chelator. Each new chelator should be tested against the different forms of iron oxyhydroxide. It may turn out that identifiably different groups of patients may need different chelators. It may even be possible to design chelators that are tissue specific so that organs with the highest loading of the more toxic forms of iron oxide are targeted, thus reducing the overall dosage of chelator required.

In terms of clinical management of iron overload diseases, awareness of the differential toxicities of the different types of iron oxide deposit and their locations within the body may lead to a more informed assessment of the dosage of chelator required thus minimizing the dose while optimizing the protection against iron mediated cell damage reactions in the tissues. Although the work presented here has assessed the relative toxicities of two forms of iron oxyhydroxide deposit, in so far as their relative solubilities are expected to reflect their toxicities, it will also be necessary to evaluate the different forms of iron oxyhydroxide in terms of their ability to generate free radicals. It is the generation of free radicals that is the critical step in cell damaging reactions [42,171–174].

This chapter has concentrated on two particular types of iron oxyhydroxide deposit in two groups of thalassemic patients. However, there are several other iron overload syndromes, such as genetic (primary) hemochromatosis, Bantu siderosis and transfusion dependent conditions other than thalassemia. Thus, further research will be required to gain statistically significant data to determine whether there are chemical speciation differences in the iron deposits related to each of these conditions. A problem often encountered in such research is the availability of tissue samples. However, it may be possible to use wax embedded archival specimens for study since it is mainly the chemical form of iron that is of interest. A recent study has looked at the effect of standard histological processing on the form of iron in iron loaded human tissues [175]. The study showed that it is feasible to use archived fixed and embedded human tissue samples for studies aimed at gauging the relative fraction of goethite like hemosiderin present in tissues.

In summary, it can be concluded that pathological iron biomineralization in humans is a widespread and complex phenomenon with direct implications for the understanding and management of iron overload diseases. It is anticipated that further research into these phenomena, much of which will require new state of the art technologies, will result in improved clinical management of patients with iron overload conditions.

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With kind thanks to Ann Lloyd-Griffiths for compilation of this index